AD_____

Award Number: W81XWH-10-1-0679

TITLE: DOES LACTATION MITIGATE TRIPLE NEGATIVE/BASAL BREAST CANCER PROGRESSION?

PRINCIPAL INVESTIGATOR: Tanya Russell, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Anschutz Medical Campus
Aurora, CO 80045

REPORT DATE: September 2012

TYPE OF REPORT: Revised Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2012	Revised Annual Summary	1 September 2011 – 31 August 2012

4. TITLE AND SUBTITLE

DOES LACTATION MITIGATE TRIPLE NEGATIVE/BASAL BREAST CANCER PROGRESSION?

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0679

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Tanya Russell

E-Mail: tanya.russell@ucdenver.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Colorado Anschutz Medical Campus
Aurora, CO 80045

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Young Black women develop and die more from aggressive forms of breast cancer compared to young White women. Recent data show increased risk of basal-like breast cancer with increased childbearing in Black women. Breast cancers associated with a recent pregnancy are more likely to be metastatic. We predict that aggressive breast cancers are promoted by a recent pregnancy. We developed a murine mammary intraductal model to examine effects of host reproductive status on the tumor suppressive myoepithelial cell layer with respect to tumor progression. The loss of p63 was identified as an early indicator of compromised myoepithelium. Further, our data suggest that the myoepithelial cell layer may be preferentially compromised by t umors formed in postpartum involuting mammary glands.Our preclinical model of human breast cancer provides a rigorous approach to study effects of reproductive state on DCIS progression. Our model could aid research of early disease progression, a requisite for research focused on breast cancer prevention and inhibition of local invasion. Further, this model may provide a unique opportunity to address and study tumor growth disparities among ethnically diverse women.

15. SUBJECT TERMS
Triple negative/basal breast cancer, pregnancy-associated breast cancer, young African American women, pregnancy, lactation, myoepithelial cell layer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	63	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Young African American women have an increased risk of developing aggressive forms of breast cancer (i.e. triple negative/basal-like) than young non-Hispanic white women. Recent epidemiological data show increased risk of basal-like breast cancer with increased childbearing in African American women [1, 2]. Breast cancers associated with a recent pregnancy (pregnancy-associated breast cancer) are more likely to be metastatic [3]. **We predict that the triple negative/basal-like breast cancer subtype is promoted by a recent pregnancy, accounting in part, for the poor prognosis of young African American breast cancer patients.**

We have previously reported the development of our murine intraductal mammary model [4, 5] to examine the effect of host reproductive status on the progression of early stage human breast cancer. Our model delivers human mammary tumor cells (MCF10ADCIS.com and HCC70 cell lines) directly through the intact mouse teat into the correct anatomical location for ductal carcinoma in situ (DCIS) without surgical manipulations. Using the MCF10ADCIS.com model to assess the effects of host reproductive status on DCIS progression, we found that DCIS progression to locally invasive disease occurred with progressive loss of myoepithelial cell differentiation markers. The loss of p63 was identified as an early indicator of compromised myoepithelium. Further, our data suggest that the protective myoepithelial cell layer may be preferentially compromised by tumors formed in postpartum involuting mammary glands but maintained by pregnancy.

Our murine mammary intraductal model of human breast cancer provides a rigorous approach to study early stage-tumor progression, and is well suited to study the effect of the host reproductive state on DCIS progression. Since occult tumors in women develop within ducts, we propose that this teat injection model will aid research of early disease progression, a requisite for research focused on breast cancer prevention and inhibition of local invasion. Further, this model may provide a unique opportunity to address and study tumor growth disparities among African American and non-Hispanic white women.

BODY

STATEMENT OF WORK AND ACCOMPLISHMENTS

Specific Aim 1 – Determine if pregnancy and/or involution, in the absence of lactation, confer tumor promotion of triple negative/basal breast cancer.

Task 1 – Develop intraductal xenograft mouse model of triple negative/basal breast cancer during pregnancy.

Months 1-12

1a. Randomize 48 SCID mice, 5-weeks of age, into 2 groups (nulliparous control and day 19 of gestation) with 12 mice/group for each triple negative/basal cell line (MCF10ADCIS.com and MDA-MB-157). Task completed for MCF10ADCIS.com cell line.

1b. Intraductally inject either MCF10ADCIS.com or MDA-MB-157 into left and right 3rd and 4th mammary glands of all mice at age 5 weeks. Task completed for MCF10ADCIS.com cell line.

1c. Breed mice except the nulliparous controls at 10 weeks of age. Task completed MCF10ADCIS.com cell line.

1d. Euthanize pregnant mice on day 19 of gestation along with age-matched nulliparous controls. Tumor "age" at this stage is 8 weeks. Task completed for MCF10ADCIS.com cell line.

1e. Harvest the left and right 3rd and 4th mammary glands 8 weeks post-injection and fix in formalin for histology. Task completed for MCF10ADCIS.com cell line.

Months 12-24

1f. Submit manuscript characterizing the effect of pregnancy and involution on early stages of tumor progression (currently in progress).

1g. Submit manuscript describing the technical aspects of the intraductal injection method (currently in progress). Task completed (resubmission in progress).

1h. Continue characterizing changes in the expression of myoepithelial cell markers with respect to tumor progression in the pregnant and nulliparous host environments (currently in progress).

1i. Develop intraductal xenograft mouse model of triple negative/basal breast cancer during pregnancy using the MDA-MB-157 cell line. Currently in progress using HCC70 cell line (see explanation in Major Updates for Tasks 1a-1e).

Major Updates for Tasks 1a – 1e:

1. In our intraductal preclincal model of human breast cancer, MCF10ADCIS.com cells undergo evolution from stages of DCIS development (hyperplasia, solid, cribriform, papillary, and comedo) to invasive lesions in both the nulliparous and pregnant host environments as assessed by hemotoxylin and eosin histology (Figure 1).

2. Tumor burden was not increased in the pregnant group compared to their respective nulliparous controls (Figure 2A). Ki67 proliferative index (Figure 2B) is increased in tumors from the nulliparous group compared to normal cells ($^{\dagger}p = 1.956E-06$). Interestingly, there was no difference in Ki67 proliferative index between tumors and normal cells in the pregnant group ($p = 0.1256$). There was a slight but significant decrease in Ki67 proliferative index in tumors from the pregnant group compared to tumors from their respective nulliparous controls ($^{\dagger\dagger}p = 0.0441$). As expected, Ki67 proliferative index was much higher in normal cells from the pregnant group compared to nulliparous controls ($^{\dagger\dagger\dagger}p = 1.407E-05$).

3. Although MCF10ADCIS.com cells were initially characterized as being triple negative [6], some MCF10ADCIS.com tumors re-express estrogen receptor (ER) in vivo (Figure 3A). There was a slightly lower but significant ($*p = 0.0697$) expression of ER in the pregnant group compared to their respective nulliparous controls (Figure 3B). Thus, tumors in the pregnant group appear to be less proliferative and have slightly lower ER expression than tumors in their respective nulliparous controls, suggesting that the hormonal milieu of pregnancy may have an effect on certain mechanisms of tumor progression.

4. In the pregnant host environment myoepithelial cells surrounding DCIS lesions have higher expression of smooth muscle actin and calponin compared to their respective nulliparous controls, suggesting a more differentiated phenotype. Myoepithelial cell expression of p63, a putative tumor suppressor, is lost early during DCIS progression independent of group (Figure 4 and Table 1).

Major Updates for Tasks 1f – 1i, Year 2:

1. Submitted manuscript describing the technical aspects of the intraductal injection method (see Appendices).

2. Levels of Cyclooxigenase-2 (COX2), an enzyme responsible for the formation of prostaglandins during inflammatory response, were not increased in the pregnant group compared to respective nulliparous controls (Figure 1).

3. Using our intraductal preclinical model of human breast cancer, we obtained and tested another other triple negative breast cancer cell lines, HCC70 (Figure 2A), in addition to the proposed MDA-MB-157 cell line (Figure 2B). The HCC70 cell line provided the most robust results, developing tumors throughout the mouse mammary gland and displaying certain characteristics of human DCIS in vivo.

4. In an effort to understand the role of the mammary myoepithelial cell layer with respect to tumor progression, we performed a detailed analysis on early stages of tumor progression in our mammary intraductal model. Nulliparous mouse mammary glands were intraductally injected with MCF10ADCIS.com cells and mammary glands were harvested 96 hours, 4 weeks, or 10 weeks post injection. Mammary ducts contain more normal (3-4 cell tumor cell bolus) and hyperplastic lesions at 96h post-injection (Figure 6A). By 4 weeks post injection, the mammary gland contains a heterogeneous display of tumor progression but has shifted to more DCIS lesions (Figure 6B). At 10 weeks post injection, tumor progression has shifted to more DCIS and invasive lesions (Figure 6C).

5. Assessed the myopepithelial cell layer during early stages of tumor progression (Figure 7). The myoepithelial cell layer is compromised early during DCIS progression of MCF10ADCIS.com tumors; the tumor suppressor p63 is lost by 96 hours post injection, while smooth muscle actin (SMA) and calponin are intact. By 4 weeks post injection, all myoepithelial cell markers are markedly decreased (Figure 7).

6. Confirmed our initial data from Year 1 with a more detailed analysis on the effect of pregnancy on the myoepithelial cell layer (Figure 8). Our data suggests that the myoepithelial cell layer around tumors may not be compromised during pregnancy, suggesting that pregnancy may maintain its tumor suppressive phenotype.

Proposed Work for Tasks 1a – 1i, Year Three:

1. Resubmit manuscript describing the technical aspects of the intraductal injection method (currently in progress).

2. Submit manuscript characterizing the effect of pregnancy and involution on early stages of tumor progression (currently in progress).

3. Repeat and further analyze the luminal A cell line (MCF7 and T47D) injection experiment to further assess the effect of pregnancy on tumor progression of hormone receptor positive cells.

4. Develop intraductal xenograft mouse model of triple negative/basal breast cancer during pregnancy using the HCC70 cell line.

5. Continue characterizing changes in the expression of myoepithelial cell markers with respect to tumor progression in the pregnant and nulliparous host environments.

Months 1-12

1f. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed for MCF10ADCIS.com cell line.

Major Updates for Task 1f:

1. We currently have paraffin embedded tissue from liver, lung, kidney and brain from both nulliparous and pregnant groups and lung serial sections cut from nulliparous and pregnant groups.

2. We have also collected frozen tissue from liver, lung, kidney and brain from both nulliparous and pregnant groups to assess metastases by QRT-PCR analysis.

Months 12-24

1j. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed for MCF10ADCIS.com cell line.

Major Updates for Task 1j:

We did not detect lung (or liver?) metastasis at 4 weeks post-intraductal injection in the pregnant group.

Proposed Work for Task 1j, Year Three:

Since it appears that pregnancy may have a protective effect on tumor progression, no further work with respect to metastasis is proposed for Year Three.

Task 1A –Develop intraductal xenograft mouse model of luminal A breast cancer during pregnancy.

Months 12-24

1a. Randomize 9 SCID mice, 5-weeks of age, into 3 groups (no pellet control, estrogen pellet, pregnancy/no pellet) with 3 mice/group for each luminal A cell line (MCF7.com and T47D). Task completed.

1b. Intraductally inject either MCF7 or T47D into left and right 3rd and 4th mammary glands of all mice at age 5 weeks and insert estrogen pellets. Task completed.

1c. Breed mice except the nulliparous controls at 10 weeks of age. Task completed.

1d. Euthanize pregnant mice on day 19 of gestation along with age-matched nulliparous controls. Tumor "age" at this stage is 8 weeks. Task completed.

1e. Harvest the left and right 3rd and 4th mammary glands 8 weeks post-injection and fix in formalin for histology. Task completed.

1f. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed.

Major Updates for Tasks 1A, 1a-1f:

1. We determined if lack (or minimal expression) of both estrogen receptor (ER) and progesterone receptor (PR) in MCF10ADCIS.com cells, and subsequent lack of response to pregnancy hormones, attributed to the lack of tumor burden increase during pregnancy by injecting luminal A cell lines (MCF7 and T47D in the presence and absence of estrogen supplementation (see University of Colorado IACUC amendment approval letter in Appendices).

2. All groups of female SCID mice were injected with either or both MCF7 cells (right 4th inguinal mammary gland) and T47D cells (left 4th inguinal mammary gland) and divided into three groups: Group 1 – no pellet control; Group 2 – estrogen pellet; Group 3 – pregnancy/lactation, no estrogen pellet.

a. Group 2 received estrogen pellets (0.72 mg/pellet) at the time of injection.
b. Group 3 did not receive estrogen pellets and were bred 5 days post-injection.
c. Mammary glands from all groups were harvested at the same time as Group 3 (~3 weeks post injection), which were euthanized 2 days post-lactation.

3. Initial hemotoxylin and eosin (H&E) and fluorescent in situ hybridization anaylsis (FISH) analysis showed no T47D tumors in the mammary glands from Group 1 (Figure 3).

4. No T47D tumors were evident in mammary glands from Group 2 (Figure 4A), whereas MCF7 tumors formed in mammary glands from Group 2 (Figure 4B). Although no T47D tumors were evident in Group 2 (Figure 4A), a few developing tumors were present in some mammary ducts (Figure 5A). Similarly, there were areas of putative MCF7 tumor development in Group 3 (Figure 5B), but they were not as prominent as those in Group 2 (Figure 4B). Along with the tumor burden and proliferation data from Year 1, these data further suggest that pregnancy has a protective effect with respect to tumor progression.

Proposed Work for Task 1A, Year Three:

Since it appears that pregnancy may have a protective effect on tumor progression, no further work with respect to luminal A breast cancer is proposed for Year Three.

Task 2 – Develop our intraductal xenograft model of pregnancy associated breast cancer to determine whether the involution microenvironment alone promotes triple negative/basal metastasis. Months 1-12

Months 1-12

1a. Randomize 48 SCID mice, 5-weeks of age, into 2 groups (nulliparous control and day 19 of gestation) with 12 mice/group for each triple negative/basal cell line (MCF10ADCIS.com and MDA-MB-157). Task completed for MCF10ADCIS.com cell line.

1b. Breed mice at 5 weeks of age except the nulliparous controls. After parturition, normalize pups to 8, and wean at 9 days of parturition to initiate involution. Task completed for MCF10ADCIS.com cell line.

1c. Intraductally inject either MCF10ADCIS.com or MDA-MB-157 into left and right 3rd and 4th mammary glands of mice 2 days post-weaning (also inject age-matched nulliparous controls). Task completed for MCF10ADCIS.com cell line.

1d. Euthanize all mice 8 weeks post-injection. Tumor "age" at this stage is 8 weeks. Task completed for MCF10ADCIS.com cell line.

1e. Harvest the left and right 3rd and 4th mammary glands 8 weeks post-injection and fix in formalin for histology. Task completed for MCF10ADCIS.com cell line.

Months 12-24

1f.. Submit manuscript characterizing the effect of pregnancy and involution on early stages of tumor progression (currently in progress).1g.Continue characterizing changes in the expression of myoepithelial cell markers with respect to tumor progression in the involution and nulliparous host environments. Task completed for MCF10ADCIS.com cell line.

1h.Develop intraductal xenograft mouse model of triple negative/basal breast cancer during involution using the HCC70 cell line. Currently in progress (See Major Updates for Task 1 for explanation of new cell line addition).

Major Updates for Task 2 (1a – 1e):

1. In our intraductal preclinical model of human breast cancer, MCF10ADCIS.com cells undergo evolution from stages of DCIS development (hyperplasia, solid, cribriform, papillary, and comedo) to invasive lesions in both the nulliparous and involution host environments as assessed by hemotoxylin and eosin histology (Figure 5A) and fluorescent in situ hybridization analyses (Figure 5B).

2. Tumor burden is greater in the involution group compared to their respective nulliparous controls (Figure 6A). Ki67 proliferative index (Figure 6B) is increased in tumors from both the nulliparous and involution groups compared to normal cells ($^{*}p = 5.859E-08$ and 1.082E-07, respectively). There was no significant difference in Ki67 proliferative index between tumors in the nulliparous group compared to the involution group ($p = 0.1737$). However, there was a decrease in Ki67 proliferative index in normal cells from the involution group compared to normal cells from their respective nulliparous controls ($^{**}p = 0.021$).

3. There was no difference in ER expression in tumors from the involution group compared to their respective nulliparous controls (*p = 0.3009) (Figure 7).

4. In myoepithelial cells surrounding post-partum involution group DCIS lesions, smooth muscle actin expression is high, but calponin is lost at a higher frequency, suggesting a less differentiated phenotype. Myoepithelial cell expression of p63, a putative tumor suppressor, is lost early during DCIS progression independent of group (Figure 8 and Table 2). Taken together with the pregnancy data (Figure 4 and Table 1) SMA positivity remains relatively stable, suggesting that the actin biostructure may be a later barrier broken in tumor progression. In contrast, p63 positivity appears to be lost first in all tested microenvironments and calponin positivity appears to lost preferentially in the post-partum involution environment. These data may reflect key differences in the influence of these reproductive states on tumor progression.

Major Updates for Task 2 (1f – 1h), Year 2:

1. Levels of Cyclooxigenase-2 (COX2), an enzyme responsible for the formation of prostaglandins during inflammatory response, were increased in the involution group compared to respective nulliparous controls (Figure 9).

2. Developed intraductal xenograft mouse model of triple negative/basal breast cancer during involution using the HCC70 cell line (currently in progess).

3. Initiated an additional objective to analyze tumor cell escape during involution. In our current involution model, tumor cells are injected during early stages of involution (Day 2) when the mammary gland is actively remodeling. This process could allow tumor cells to readily escape mammary ducts. We assessed whether "pre-existing" MCF10ADCIS.com tumor cells have the ability to escape the mammary ducts at the onset of involution (currently in progress).

    a. Two groups of female SCID mice were injected with MCF10ADCIS.com cells (both right and left 4th inguinal mammary glands): Group 1 – nulliparous control; Group 2 – early involution group.
    b. Group 2 females were bred 5 days post-injection. Pup litters were normalized to eight pups 1 day postpartum.
    c. Mammary glands from Group 2 were harvested at involution day 6. Mammary glands from Group 1 were harvested at the same time.

4. Confirmed our initial data from Year 1 with a more detailed analysis on the effect of involution on the myoepithelial cell layer (Figure 10). Our data suggests that the protective myoepithelial cell layer may be compromised by tumors formed in post-partum involution mammary glands.

Proposed Work for Task 2 (1f – 1h), Year Three:

1. Analyze mammary glands from the tumor cell escape during involution experiment (see Major Update number 3) using IHC and FISH (currently in progress).

2. Submit manuscript characterizing the effect of pregnancy and involution on early stages of tumor progression (currently in progress).

Months 1-12

1f. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed for MCF10ADCIS.com cell line.

Major Updates for Task 2 (1f):

1. We did not detect lung metastasis at 4 weeks post-intraductal injection in neither the involution nor nulliparous groups by QRT-PCR (Figure 9). We also did not detect lung or liver metastasis at 8 weeks post-intraductal injection by FISH analysis (Figure 10).

Proposed Work for Task 2 (1f), Year Two:

1. Determine if metastasis occurs in the intraductal injection model by increasing "tumor age" in the involution and nulliparous host environments.

Months 12-24

1i. Determine if metastasis occurs in the intraductal injection model by increasing "tumor age" in the involution and nulliparous host environments.

Major Updates for Task 2 (1i):
Although some large tumors formed ($2cm^3$), we did not detect lung or liver metastasis at 10-12 weeks post-intraductal injection.

Proposed Work for Task 2 (1i), Year Three:
No further work with respect to metastasis is proposed for Year Three.

Task 3 – Develop a two-hit pregnancy/involution tumor promotional intraductal xenograft model in the absence of lactation. Months 12-24

1a. Randomize 48 SCID mice, 8-weeks of age, into 2 groups (nulliparous control and day 19 of gestation) with 12 mice/group for each triple negative/basal cell line (MCF10ADCIS.com and MDA-MB-157). Task completed for MCF10ADCIS.com cell line with 18 mice.

1b. Breed mice at 8 weeks of age except the nulliparous controls. Task completed for MCF10ADCIS.com cell line with 18 mice.

1c. Intraductally inject either MCF10ADCIS.com or MDA-MB-157 into left and right 3rd and 4th mammary glands of mice at day 1 of pregnancy (also inject age-matched nulliparous controls). Task completed for MCF10ADCIS.com cell line with 18 mice.

1d. Remove pups at parturition and allow mammary glands to involute 1 week and fully regress 4 weeks. Task completed for MCF10ADCIS.com cell line with 18 mice.

1e. Euthanize all mice at 4 weeks full-regression. Tumor "age" at this stage is 8 weeks. Task completed for MCF10ADCIS.com cell line with 18 mice.

1f. Harvest the left and right 3rd and 4th mammary glands and fix in formalin for histology. Task completed for MCF10ADCIS.com cell line with 18 mice.

1g. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed for MCF10ADCIS.com cell line with 18 mice.

Major Updates for Task 3:

1. Due to technical difficulties with the particular batch of MCF10ADCIS.com cells, we did not get a sufficient tumor take rate. Only 3 of the 18 injected mammary glands developed tumors in this particular experiment.

Proposed Work for Task 3, Year Two:
1. Complete intraductal injections with remaining proposed number of mice during pregnancy and involution in the absence of lactation.

2. Evaluate tumor latency, growth, burden and multifocal nature of tumor.

2. Characterize changes in the expression of myoepithelial cell markers with respect to tumor progression in absence of lactation.
3. Analyze evidence of metastasis by QRT-PCR, IHC and FISH.

Task 4 – Manuscript Preparation. Months 18-24

Proposed Work for Task 4, Year Two:

1. Submit manuscript characterizing the effect of pregnancy and involution in the absence of lactation on tumor progression (currently in progress)

Specific Aim 2 – Determine if lactation mitigates promotional effects of pregnancy/involution on triple negative/basal-like breast tumor progression.

Task 1 – Develop an intraductal xenograft mouse model of triple negative/basal-like breast cancer to determine if length of lactation mitigates tumor promotion. Months 24-36

1a. Randomize 60 SCID mice, 8-weeks of age, into 5 groups of increasing lengths of lactation with12 mice/group for each triple negative/basal cell line (MCF10ADCIS.com and MDA-MB-157). Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice), Group 5 (7 mice), and nulliparous controls (6 mice).

Group #	Tumor cell Injection	Gestation (weeks)	Lactation (weeks)	Post-partum involution (weeks)	Full Regression (weeks)	End of study tumor "age" (weeks)
1	P1	3	0	1	4	8
2	P1	3	0.28 (2 days)	1	4	8
3	P1	3	1.5	1	2.5	8
4	P1	3	3	1	1	8
5	P1	3	4	1	0	8

1b. Breed mice at 8 weeks of age except the nulliparous controls. Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice) and Group 5 (7 mice).

1c. Intraductally inject either MCF10ADCIS.com or MDA-MB-157 into left and right 3rd and 4th mammary glands of mice at day 1 of pregnancy (also inject age-matched nulliparous controls. Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice), Group 5 (7 mice), and nulliparous controls (6 mice).

1d. Euthanize animals at various stages of full gland regression for tumor "age" to be 8 weeks throughout entire study. Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice), Group 5 (7 mice), and nulliparous controls (6 mice).

1e. Harvest the left and right 3rd and 4th mammary glands and fix in formalin for histology. Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice), Group 5 (7 mice), and nulliparous controls (6 mice).

1f. Harvest the liver, lung, kidney and brain tissues for determination of metastatic lesions by both IHC and PCR based techniques. Task completed for MCF10ADCIS.com cell line for Group 1 (7 mice), Group 5 (7 mice), and nulliparous controls (6 mice).

Major Updates for Task 3:

1. Initiated H&E analysis on number of mammary glands developed within each group (currently in progress).

2. Initiated IHC analysis on myoepithelial cell markers p63, SMA, and calponin (currently in progress)

Proposed Work for Task 1, Year Three:

1. Complete intraductal injection experiments for Groups 2-4 with MCF10ADCIS.com cells.
2. Develop an intraductal model to determine if lactation mitigates tumor progression with HCC70 tumor cells.

Task 2 – Manuscript Preparation. Months 30-36

Task 2, Aim 2 Progress in First Year of Funding (1a – 1f), Year Two: Not yet initiated.

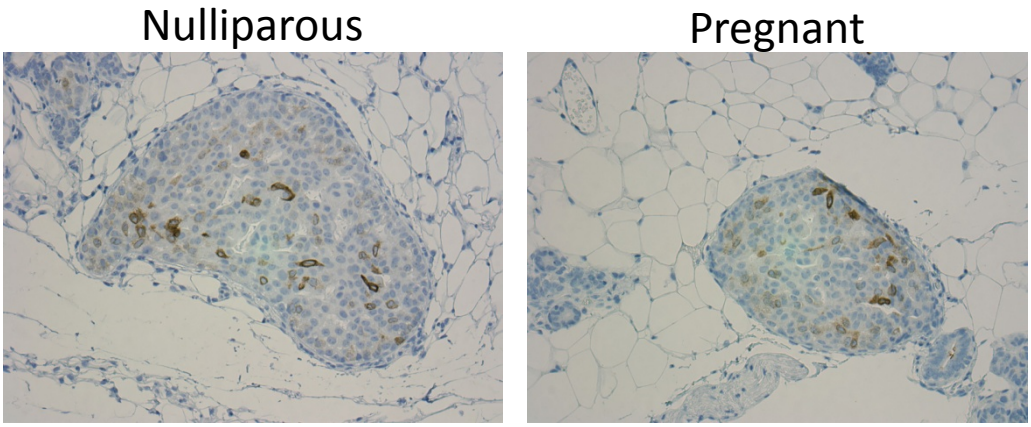
Proposed Work for Task 2, Year Three:

1. Begin outline of manuscript.

SUPPORTING DATA

FIGURES

1A



1B

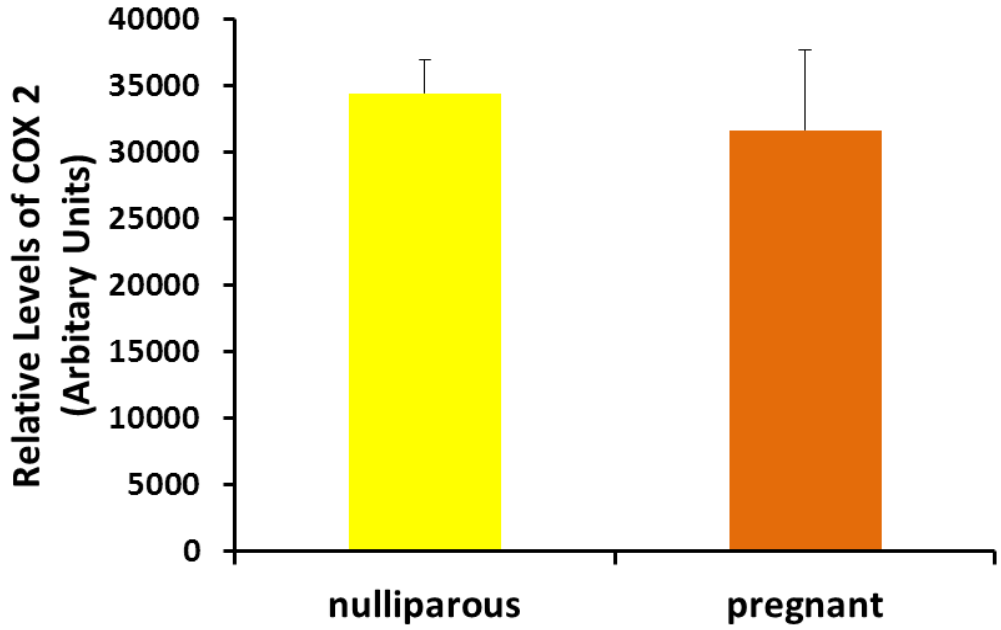
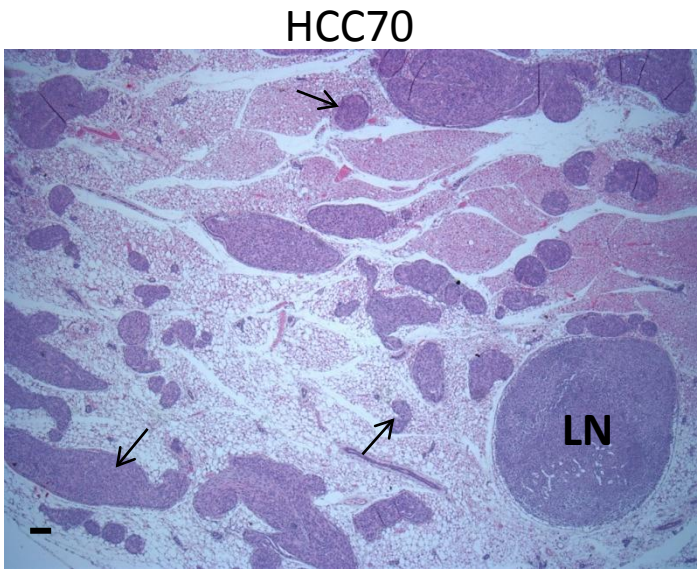


Figure 1. Effect of pregnancy on COX2 expression. (1A) IHC analysis shows COX2 (brown) expression in MCF10ADCIS.com tumors in nulliparous (left panel) and pregnant (right panel) mouse mammary glands. (1B) COX2 expression in MCF10ADCIS.com cells in the pregnant group and their respective nulliparous group are similar. Error bars = SEM.

2A



2B

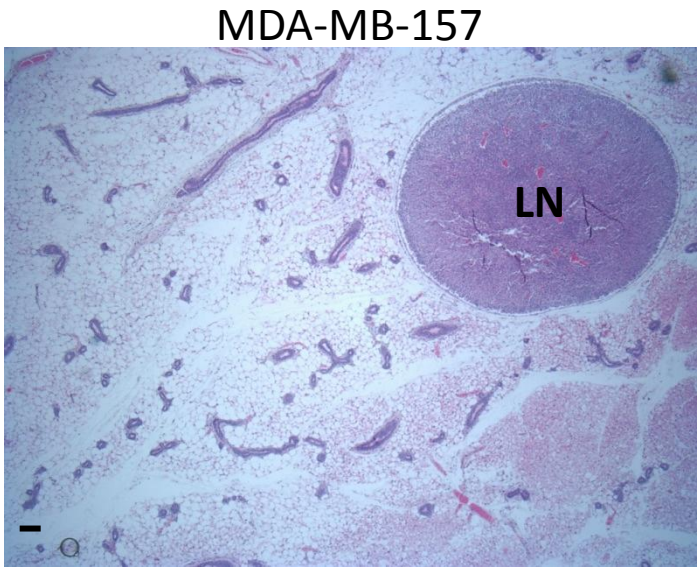


Figure 2. Hemotoxylin and eosin staining of mouse mammary glands intraductally injected with MDA-MB-157 or HCC70 tumor cells. Mouse mammary glands were harvested 4 weeks post intraductal injection. (2A) Robust tumor formation was seen in mammary glands injected with HCC70 cells (arrows). (2B) No tumors were evident in mammary glands injected with MDA-MB-157 cells. LN = lymph node. Scale bars = 50 um. Magnification = 2.5X.

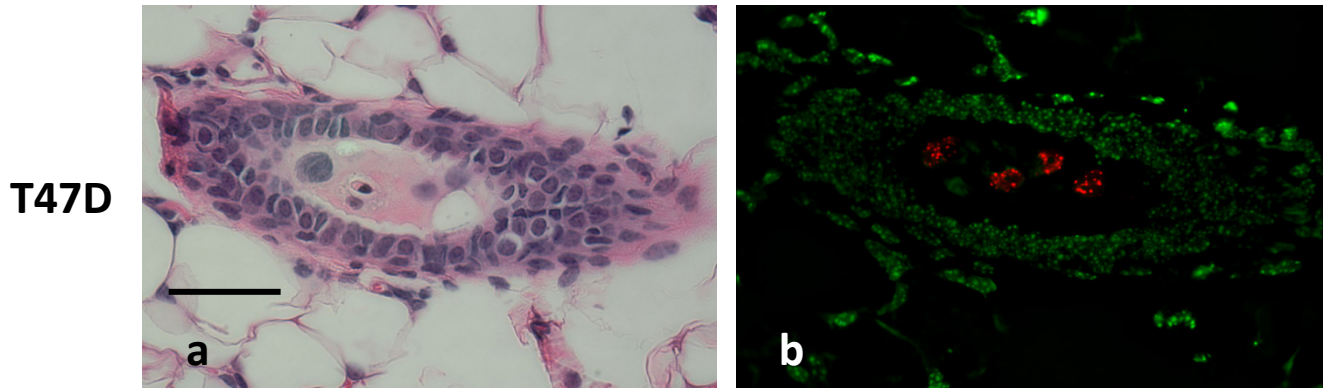
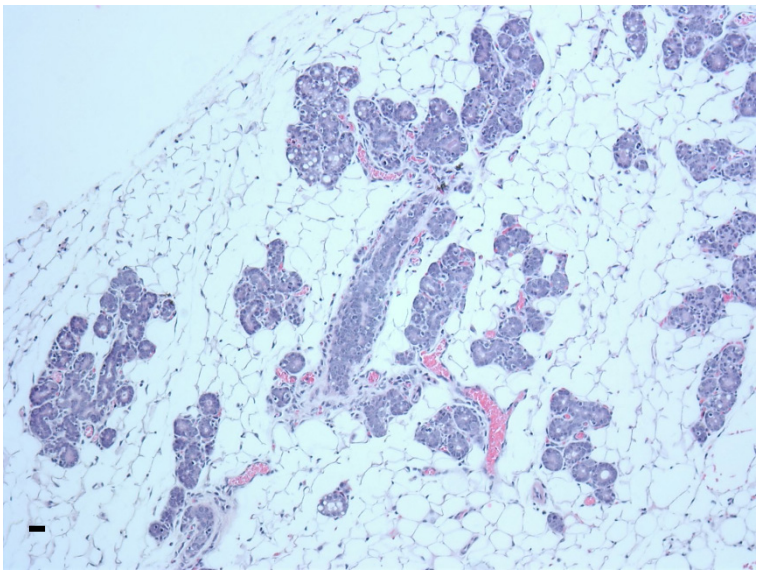


Figure 3. Hemotoxylin and eosin (H&E) staining and fluorescence in situ hybridization (FISH) analysis of nulliparous mouse mammary glands intraductally injected with a luminal A (T47D) breast cancer cell line. At 4 weeks post mammary intraductal injection in the absence of estrogen supplementation, T47D cells did not form tumors (a). FISH analysis for COT-1 DNA (red = human COT-1; green = mouse COT-1) confirms the presence of T47D cells within mammary ducts using FISH analysis (b). Scale bar = 50 um. Magnification = 40X.

4A



4B

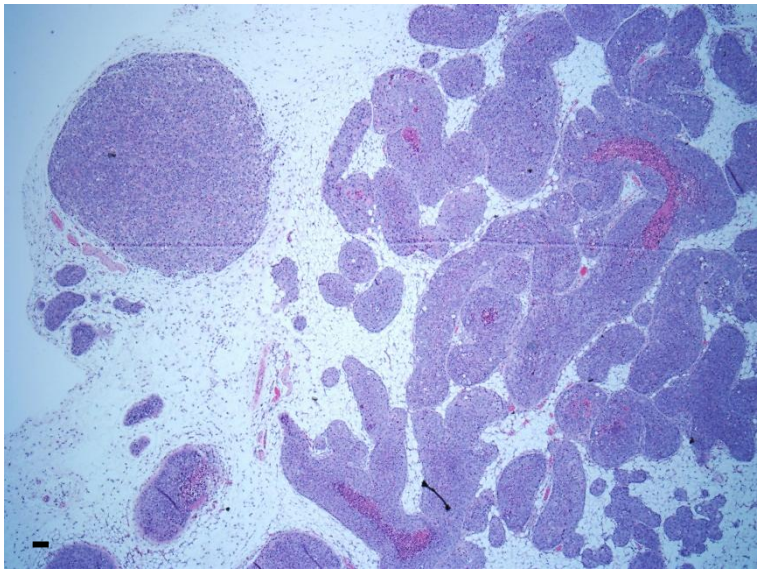
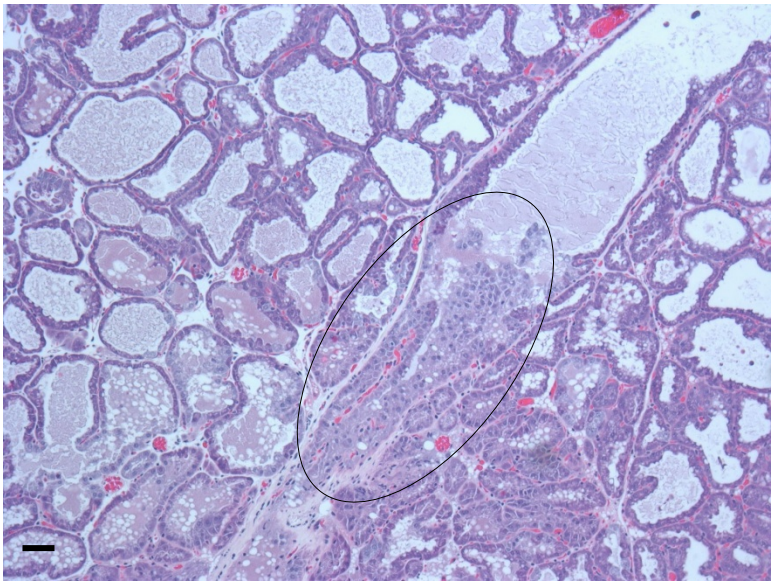


Figure 4. H&E staining of nulliparous mouse mammary glands intraductally injected with T47D and MCF7 breast cancer cell lines in the presence of estrogen supplementation. Mice were supplemented with an 0.72 mg estrogen pellet at time of intraductal injection. (4A) While estrogen supplementation stimulated a pregnant phenotype in the mammary glands, no T47D tumors were evident. (4B) MCF7 had a robust response to estrogen supplementation and formed tumors. Scale bars = 50 um. Magnification = 10X.

5A



5B

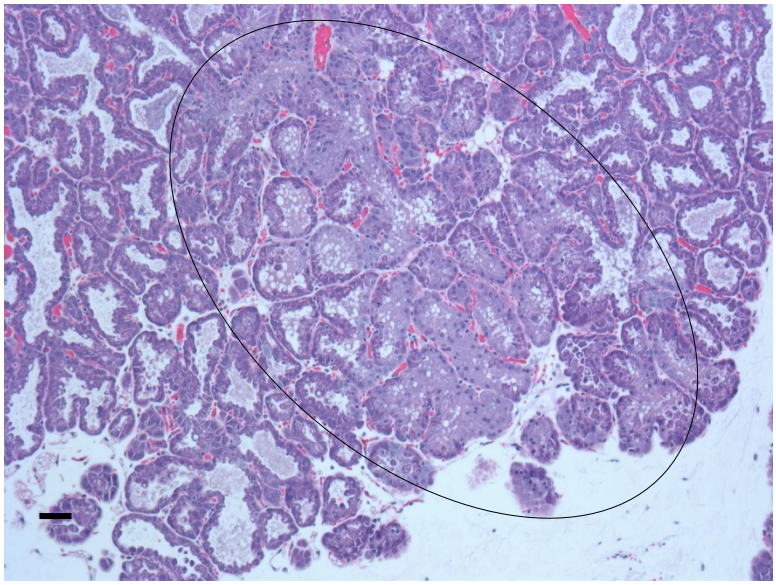
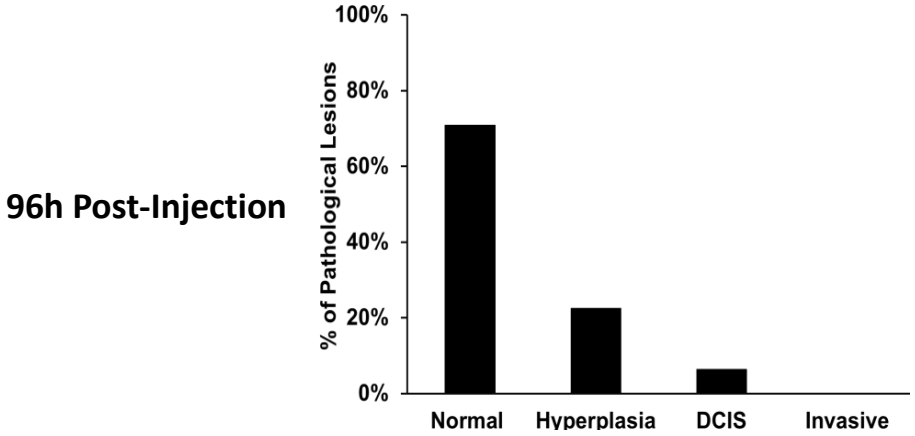
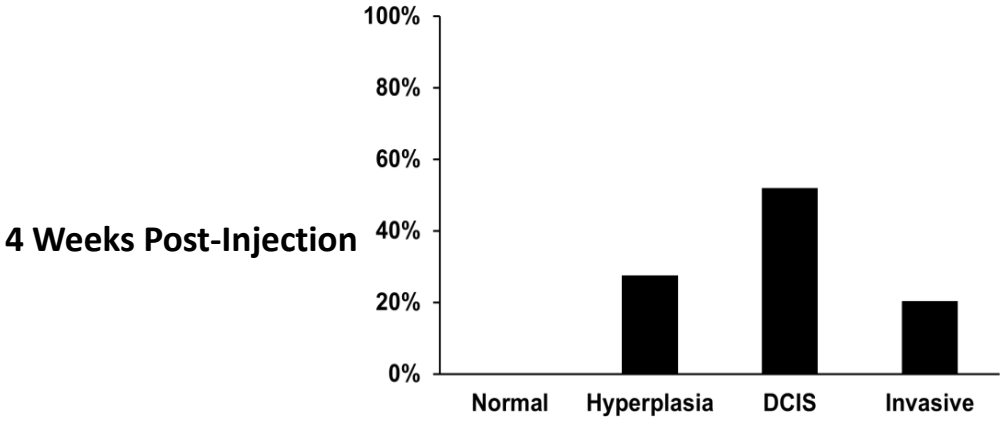


Figure 5. Effect of pregnancy on T47D and MCF7 tumor progression. (5A) T47D cells were evident in mammary ducts in mammary glands, but no distinct tumors were seen. (5B) Putative areas of MCF7 tumor development were also seen in the pregnant mammary microenvironment, but not as prominent as that seen in nulliparous glands (see Figure 4B). Scale bars = 50 um. Magnification = 10X.

6A



6B



6C

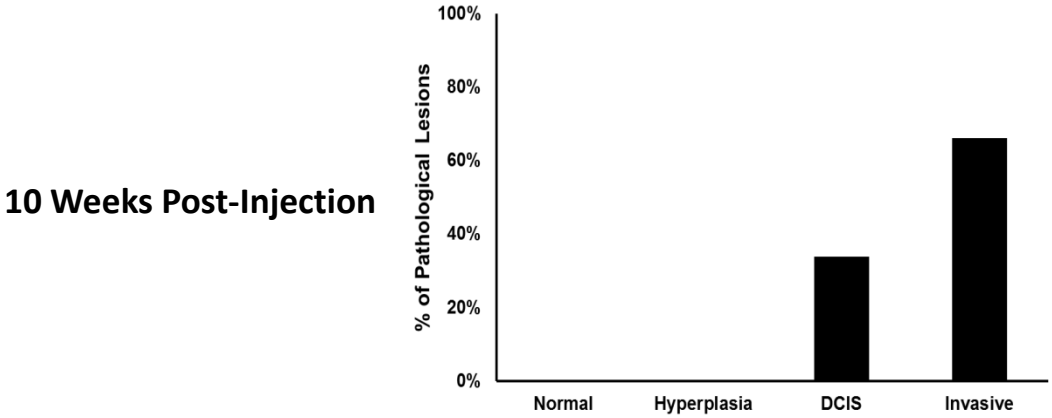


Figure 6. Quantative analysis of tumor progression in nulliparous mouse mammary glands. (6A) More hyperplastic/normal (3-4 tumor cell bolus) lesions are seen 96-hours post-injection, whereas more DCIS lesions are present 4 weeks post-injection (6B). (6C) At 10 weeks, more invasive lesions are seen.

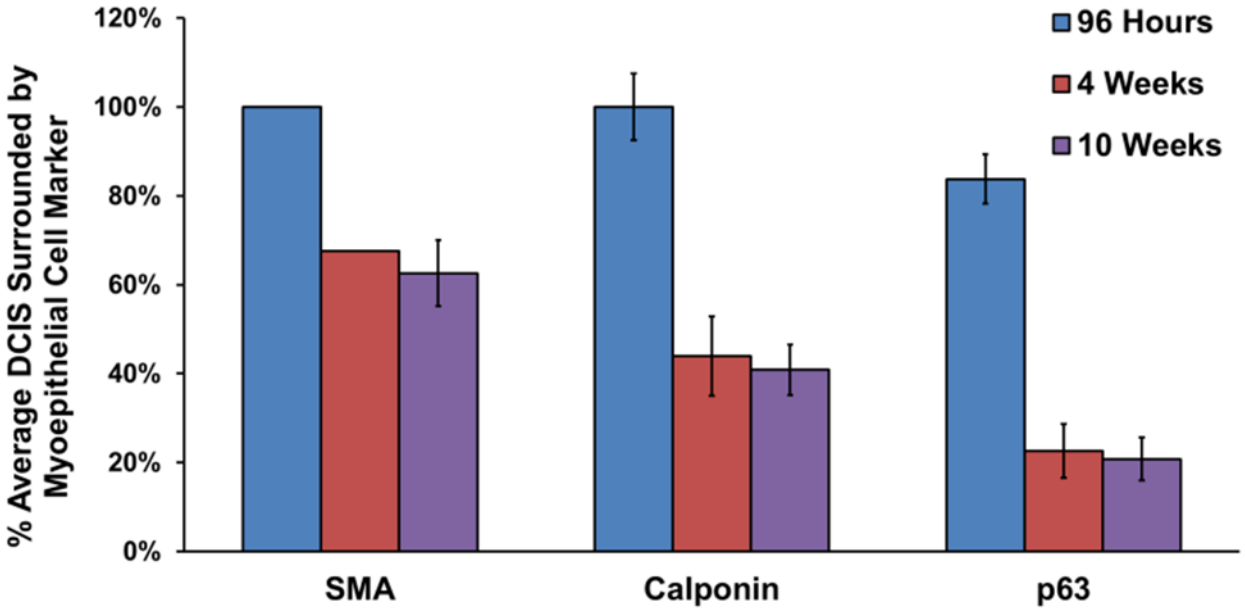


Figure 7. Anaylsis of DCIS surrounded by myoepithelial cell markers during early tumor progression. Mouse mammary glands intraductally injected with MCF10ADCIS.com cells were harvested 96 hours (blue), 4 weeks (red) or 10 weeks (purple) post injection and processed for IHC analysis of SMA, calponin, and p63. Myopepithelial cell layer is compromised early during DCIS progression of MCF10ADCIS.com tumors. Error bars = SEM.

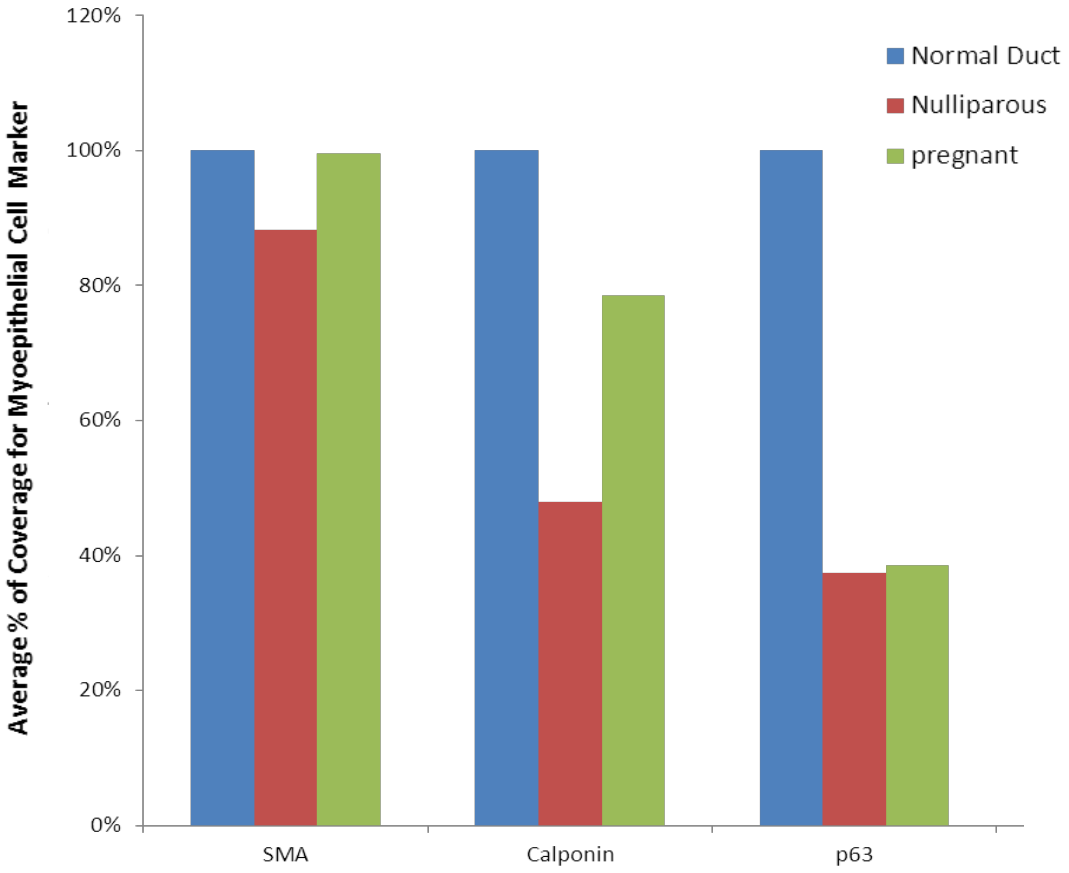


Figure 8. Progressive myoepithelial cell layer loss during pregnancy. Mouse mammary glands from nulliparous and pregnant mice were intraductally injected with MCF10ADCIS.com cells and harvested 4 weeks post injection. IHC analysis shows that SMA, calponin, and p63 surrounding normal ducts (blue) stay intact. SMA remains intact regardless of reproductive stage. Calponin and p63 surrounding DCIS in nulliparous mammary glands (red) are both decreased, while calponin surrounding DCIS in pregnant mammary glands (green) remain relatively intact.

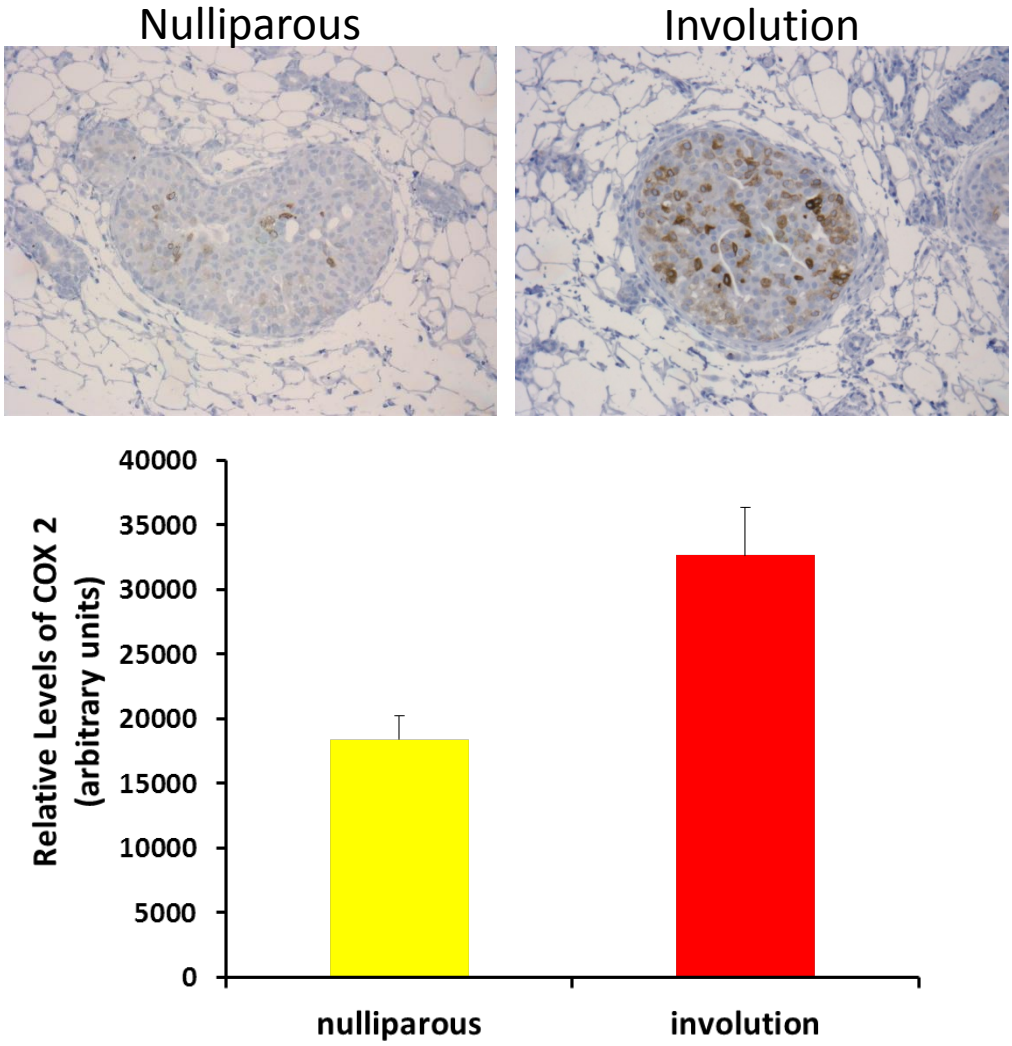


Figure 9. Effect of post-partum involution on COX2 expression in MCF10ADCIS.com tumors. (1A) IHC analysis shows COX2 (brown) expression in MCF10ADCIS.com tumors in nulliparous (left panel) and involution group (right panel) mouse mammary glands. (1B) COX2 expression in MCF10ADCIS.com cells in the involution group is higher than that of the respective nulliparous group. Error bars = SEM.

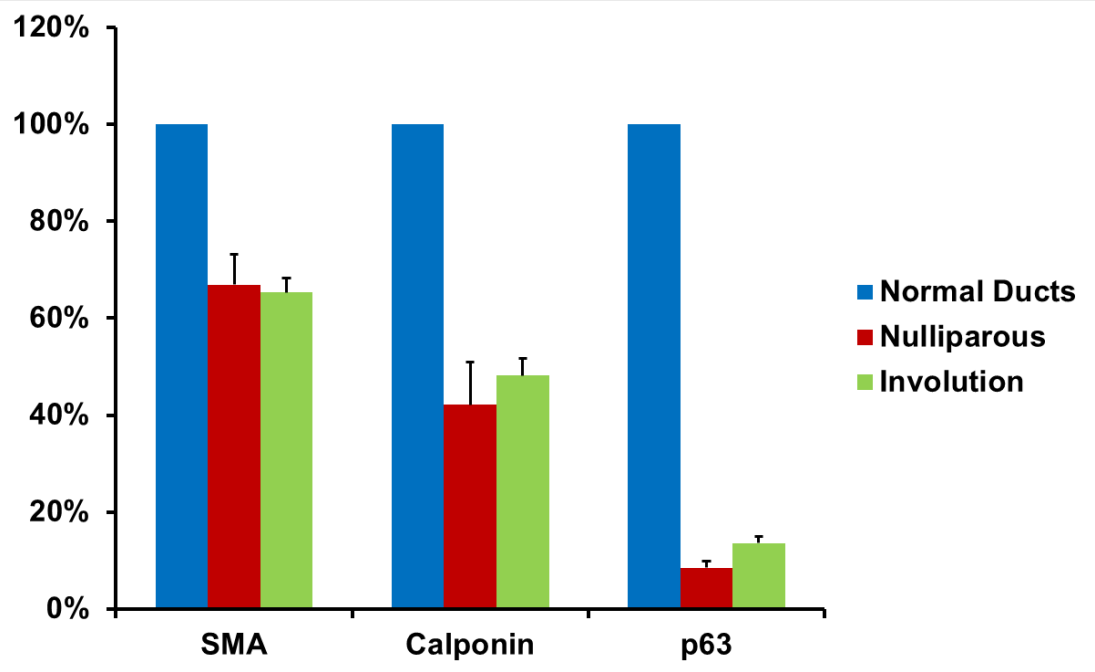


Figure 10. Progressive myoepithelial cell layer loss during post-partum involution. Mouse mammary glands were intraductally injected with MCF10ADCIS.com cells and harvested 4 weeks post injection. IHC analysis shows that SMA, calponin, and p63 surrounding normal ducts (blue) stay intact. SMA remains relatively intact surrounding tumors in nulliparous (red) and involution (green) group mammary glands. Calponin and p63 surrounding DCIS are decreased in both nulliparous (red) and involution group mammary glands (green) are decreased. Error bars = SEM.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

1. Successful development of a preclinical intraductal model of human breast cancer that minimizes wound healing programs or inflammation, which allows analysis of tumor cell progression within mammary ducts in the context of environmental changes associated with normal physiology.

2. Met the criteria for a model of human breast cancer progression, as MCF10ADCIS.com cells display histologic progression from hyperplastic alveolar nodules to invasive lesions in nulliparous, pregnant and involution hosts.

3. Evaluated tumor latency, growth, burden and multifocal nature of tumor during pregnancy and involution, showing differential effects of these reproductive states on these tumor characteristics.

4. Showed that tumor burden was not increased in the pregnant group compared to nulliparous controls, but is greater in mammary glands from the involution group compared to nulliparous controls. Tumors in the pregnant group appear to be less proliferative and have slightly lower ER expression than tumors in their respective nulliparous controls, suggesting that the hormonal milieu of pregnancy may have an effect on certain mechanisms of tumor progression.

5. Initiated characterization of the protective myoepithelial cell layer as a focal point of tumor progression. p63 positivity appears to be lost first in all tested microenvironments and calponin positivity appears to lost preferentially in the post-partum involution environment. These data may reflect key differences in the influence of these reproductive states on tumor progression.

Year 2

1. We demonstrate proof-of-principal that host physiology (i.e. pregnancy and postpartum involution) can influence DCIS progression, identifying this model as relevant for addressing key questions such as influence of host reproductive status on breast cancer progression.

2. Our model permits a rigorous evaluation of the effects of DCIS progression on molecular changes in the myoepithelium, which serves as a barrier to invasive cancer. Our data suggests that this protective layer is more compromised during post-partum involution than during pregnancy and nulliparity.
   In both environments SMA positivity remains relatively stable, suggesting that the actin biostructure may be a later barrier broken in tumor progression. p63 positivity appears to be lost first in all tested microenvironments and calponin positivity appears to lost preferentially in the post-partum involution environment. These data may reflect key differences in the influence of these reproductive states on tumor progression.

3. Our data suggests that the myoepithelial cell layer around tumors may not be compromised during pregnancy, suggesting that pregnancy may maintain the myoepithelial cell layer and thus a more tumor suppressive environment.

REPORTABLE OUTCOMES

Manuscripts:

1. **Tanya D. Russell** and Pepper Schedin. A Novel Intraductal Delivery Model that Permits Study of Host Physiology on Human Ductal Carcinoma In Situ Progression. Submitted.

Abstracts:

1. Pepper Schedin, Traci Lyons, Jenean O'Brien, Eryn Callahan, **Tanya Russell**, Holly Martinson, and Virginia Borges. Mammary gland involution drives breast cancer progression through collagen and COX-2. IABCR Breakthrough Conference, April 15-18, 2012.

2. **Tanya D. Russell,** Jaime Fornetti, and Pepper Schedin**.** Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? Department of Defense Era of Hope Conference, Orlando, Florida, August 2-5, 2011.

3. Pepper Schedin, Traci R. Lyons, Jenean O'Brien, **Tanya Russell**, Holly Martinson, Patricia J. Keely, and Virginia Borges. Postpartum mammary gland involution promotes breast tumor progression through collagen and COX-2 and is inhibited by NSAIDS. Department of Defense Era of Hope Conference, August 2-5, 2011.

4. V.F. Borges, T. Lyons, J. O'Brien, **T. Russell**, H. Martinson, P. Keely, P.J. Schedin. The Role of Collagen and COX-2 in post-partum breast involution on the progression of pregnancy-associated breast cancer and its inhibition by NSAIDs. ASCO Annual Meeting, June 3-7, 2011.

5. **Tanya D. Russell**, Sonali Jindal, Jaime Fornetti, and Pepper Schedin. A Preclinical Mammary Intraductal Model to Study Early Stage Human Breast Cancer. 102nd AACR Annual Meeting "Innovation and Collaboration: The Path to Progress", Orlando, Florida, April 2-6, 2011.

6. **Tanya D. Russell,** Sonali Jindal, Jaime Fornetti, and Pepper Schedin. Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? University of Colorado Denver Mammary Gland Biology Retreat, Aurora, CO, January 20-21, 2011.

7. **Tanya D. Russell**, Jaime Fornetti, and Pepper Schedin. Pregnancy and Postpartum Involution Promote Tumorigenesis in a Preclinical Model for Triple Negative/Basal Breast Cancer. Third AACR Conference on "The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved", Miami, FL, September 30-October 3, 2010.

Oral Presentations:

1. **Tanya D. Russell.** Effect of Pregnancy and Partum Involution on DCIS Progression. March 1, 2012, Research in Progress, Division of Medical Oncology, University of Colorado Denver.

2. **Tanya D. Russell.** Effect of Pregnancy and Partum Involution on DCIS Progression. March 6, 2012, Postdoctoral Seminar Series, University of Colorado Denver.

3. **Tanya D. Russell.** Pregnancy and Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer. January 2011 Research in Progress, Division of Medical Oncology, University of Colorado Denver.

4. **Tanya D. Russell.** Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? January 2011 University of Colorado Denver Mammary Gland Biology Retreat, Aurora, Colorado.

5. **Tanya D. Russell.** Department of Health and Human Services (HHS) National Institutes of Health (NIH) National Cancer Institute (NCI) 2010 Cancer Research Imaging Camp June 20–25, 2010 St. Louis, MO.

Poster Presentations:

1. **Tanya D. Russell,** Jaime Fornetti, and Pepper Schedin**.** Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? October 2011 Cancer Biology Poster Session, Aurora, Colorado.

2. **Tanya D. Russell,** Jaime Fornetti, and Pepper Schedin**.** Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? August 2011 Department of Defense Era of Hope Conference, Orlando World Marriot Center, Orlando, Florida.

3. **Tanya D. Russell,** Jaime Fornetti, and Pepper Schedin**.** Does Pregnancy and Post-Partum Involution Promote Tumorigenesis in a Preclinical Model for Early Stage Human Breast Cancer? June 2011 Gordon Research Conference on Mammary Gland Biology, Salve Regina University, Newport, Rhode Island.

4. **Tanya D. Russell**, Sonali Jindal, Jaime Fornetti, and Pepper Schedin. A Preclinical Mammary Intraductal Model to Study Early Stage Human Breast Cancer. April 2011 102nd AACR Annual Meeting "Innovation and Collaboration: The Path to Progress", Orlando, Florida.

5. **Tanya D. Russell**, Jaime Fornetti, and Pepper Schedin. Pregnancy and Postpartum Involution Promote Tumorigenesis in a Preclinical Model for Triple Negative/Basal Breast Cancer. 2010 UCD Third Annual Women's Health Research Day, Aurora, Colorado.

6. **Tanya D. Russell**, Jaime Fornetti, and Pepper Schedin. Pregnancy and Postpartum Involution Promote Tumorigenesis in a Preclinical Model for Triple Negative/Basal Breast Cancer. 2010 Third AACR Conference on the "Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved", Miami, Florida.

Awards: none (Year 2)
AACR Minority Scholar in Cancer Research Award - Third AACR Conference on the "Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved", 2010

Patents and licenses: none

Development of cell lines, tissue, serum repositories: none

Informatics: none

Funding applied for based on this work: none

Employment or research opportunities:

1. University of Colorado Anschutz Medical Campus workshop, "Career Planning in the Context of Life Planning", May 4, 2011, Aurora, CO.

2. **Tanya D. Russell.** Department of Health and Human Services (HHS) National Institutes of Health (NIH) National Cancer Institute (NCI) 2010 Cancer Research Imaging Camp June 20–25, 2010 St. Louis, MO.

CONCLUSION

Our murine intraductal model of human breast cancer provides a rigorous approach to the study of early-stage breast cancer progression and demonstrates the ability to assess changes in the myoepithelium as well as the influence of the host reproductive state on DCIS progression. Understanding the role of the myoepithelial cell layer in early stage cancer may provide insight in the prevention of early invasion, and ultimately metastasis. Twenty percent of new cancers diagnosed are DCIS, and standard treatment typically involves surgery and radiation. A better understanding of the role of the myoepithelium in early stage cancers may help prevent unnecessary treatments [7] Additionally, these initial studies begin to help address the question of why breast cancer, when diagnosed within close proximity to a recent pregnancy, is more aggressive and more likely to spread and provide novel insight into the especially poor prognosis of young African American women.

REFERENCES

1. **Millikan, R.C., et al., *Epidemiology of basal-like breast cancer.* Breast Cancer Res Treat, 2008. 109(1): p. 123-39.**
2. **Palmer, J.R., et al., *Parity and lactation in relation to estrogen receptor negative breast cancer in African American women.* Cancer Epidemiol Biomarkers Prev, 2011. 20(9): p. 1883-91.**
3. **Lyons, T.R., et al., *Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2.* Nat Med, 2011. 17(9): p. 1109-15.**
4. **Russell, T.D., et al., *Transduction of the mammary epithelium with adenovirus vectors in vivo.* J Virol, 2003. 77(10): p. 5801-9.**
5. **Nguyen, D.-A.D., et al., *Intraductal injection into the mouse mammary gland.*, in *Methods in Mammary Gland Biology and Breast Cancer Research*, M.M. Ip and B.B. Asch, Editors. 2000, Kluwer Academic/Plenum: N.Y. p. 259-270.**
6. **Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. 10(6): p. 515-27.**
7. **Schnitt, S.J., et al., *Ductal carcinoma in situ (intraductal carcinoma) of the breast.* N Engl J Med, 1988. 318(14): p. 898-903.**

APPENDICES: Manuscript, IACUC Amendment Approval, and Abstracts

University of Colorado
Anschutz Medical Campus
School of Medicine

Department of Medicine
Division of Medical Oncology

Tanya D. Russell, PhD
Postdoctoral Fellow
MS 8117
12801 E. 17th Avenue
Aurora, Co 80045
Phone: 303-724-3845
Fax: 303-724-3889
Email: tanya.russell@ucdenver.edu

September 28, 2012

Dr. Lewis Chodosh, Editor-in-Chief
Breast Cancer Research
BioMed Central
236 Gray's Inn Road
London WC1X 8HB
United Kingdom

Dear Dr. Chodosh:

Enclosed please find a manuscript entitled: "A Novel Intraductal Delivery Model that Permits Study of Host Physiology on Human Ductal Carcinoma In Situ Progression" which I am submitting for exclusive consideration of publication as an article in Breast Cancer Research.

The paper demonstrates a novel, non-surgical, mammary intraductal tumor cell delivery model that provides a robust physiologic environment to study early stage human breast cancer progression with respect to changes in the myoepithelium and effects of host reproductive state. Using this model, we demonstrate gradual loss of three myoepithelial cell markers prior to the physical disruption of the myoepithelial cell layer that occurs with progression to invasive disease. To the best of our knowledge, these data represent the most robust characterization of myoepithelial cells with DCIS progression, to date. Further, using this model, we show that intraductal lesions are promoted during postpartum mammary gland involution. Previous studies have demonstrated promotion of tumor cells in direct contact with involution-stroma, which is rich in inflammatory mediators. Our unexpected result that intraductal tumor cells are also promoted during involution suggests unexpected mechanisms of cancer promotion after pregnancy. As such this paper should be of interest to a broad readership including those interested in breast cancer research, preclinical models of DCIS, and the tumor microenvironment.

We have no competing interests to declare.

Knowledgeable referees for this paper might include:

- Daniel Medina [breast cancer/xenograft models of DCIS] (dmedina@bcm.edu)
- Weston Porter [mammary gland development and breast cancer/stromal-epithelial interactions/xenograft models] (wporter@cvm.tamu.edu)
- Joyce Schroeder [breast cancer progression and invasion/transgenic models] (jschroeder@azcc.arizona.edu)
- Charlotte Kuperwasser [breast cancer progression and invasion/stromal-epithelial interactions] (charlotte.kuperwasser@tufts.edu)

Thank you for your consideration of my work. Please address all correspondence concerning this manuscript to me at the address stated above and feel free to correspond with me by e-mail (tanya.russell@ucdenver.edu).

Sincerely,

Tanya D. Russell, PhD

TITLE PAGE

Title: A Novel Intraductal Delivery Model that Permits Study of Host Physiology on Human Ductal Carcinoma In Situ Progression

Authors: Tanya D. Russell[1] and Pepper Schedin[1,2,3]

[1]School of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus, MS8117, RC-1S, 8401K, 12801 E 17th Ave, Aurora, CO, USA 80045.

[2]University of Colorado Cancer Center, Bldg 500, Suite 6004C, 13001 E 17th Place, Aurora, CO, USA 80045.

[3]AMC Cancer Research Center, 3401 Quebec Street, Suite 3000, Denver, CO

E-mail: tanya.russell@ucdenver.edu, pepper.schedin@ucdenver.edu

Corresponding Author: Tanya D. Russell, PhD, Division of Medical Oncology, Mail Stop 8117, RC-1 S, 8401K, 12801 E. 17th Avenue, UCD, Aurora, CO 80045, Phone: 303-724-3845, Fax: 303-724-3889, Email: tanya.russell@ucdenver.edu

ABSTRACT

Introduction: We describe a preclinical, mammary intraductal tumor cell delivery model that permits investigation of progression of early-stage breast cancer. Human breast tumor cells are established in the correct anatomical location for ductal carcinoma in situ (DCIS) in the absence of surgical manipulation. This model avoids wound-healing confounders and provides a more physiologic environment.

Methods: MCF10ADCIS.com cells were delivered into the intact mouse mammary teat via intraductal injection and an optimized tumor injection volume identified that did not compromise ductal integrity. To assess influence of host reproductive state on intramammary tumor progression, nulliparous or recently pregnant hosts whose mammary glands were involuting at time of tumor cell injection were utilized. Mammary glands were harvested 4 weeks post-injection, evaluated for tumor histology, tumor growth, and myoepithelial cell layer integrity, using multiple histochemical approaches.

Results: DCIS-like lesions develop throughout the gland with full representation of human DCIS histologic subtypes, which progressed through stages of atypical ductal hyperplasia, DCIS, and subsequent invasive ductal carcinoma. MCF10ADCIS.com cells incorporate into the mouse mammary ducts and form E-cadherin-based junctional complexes with each other and with neighboring normal mouse epithelial cells. Although the MCF10ADCIS.com cell line is characteristically negative for estrogen receptor (ER), progesterone receptor (PR) and Her 2 *in vitro* and *in vivo*, established intramammary tumors were noted to express ER. Progressive loss of myoepithelial cell differentiation markers is also demonstrated, identifying loss of p63 as an early indicator of compromised myoepithelium. Using this model to assess the effects of host reproductive status on DCIS progression, we found an increase in tumor incidence and burden in

the postpartum involution group compared to the nulliparous control group.

Conclusions Our murine intraductal model of human breast cancer provides a rigorous approach to the study of early-stage breast cancer progression and demonstrates the ability to assess changes in the myoepithelium as well as the influence of the host reproductive state on DCIS progression. Since mammary ducts are the primary site of occult tumors in women, we propose that this intraductal model will be a highly relevant for the study of host physiology on human breast cancer progression.

KEYWORDS

murine mammary gland, intraductal, human DCIS, tumor progression, myoepithelial cell, involution

INTRODUCTION

There is clinical evidence for histological progression of breast cancer through atypical hyperplasia, ductal carcinoma in situ (DCIS), invasive ductal carcinoma and metastasis [1]. Histopathologic and genetic progression of epithelial cancers has suggested that acquisition of metastatic potential is a relatively late event. However, microarray data demonstrate that early-stage lesions segregate into those with and without metastatic potential, highlighting the importance of early events in tumor progression [2]. Lack of relevant model systems to investigate early events in tumor cell invasion currently hinders our understanding of breast cancer progression.

The clinical definition of invasive breast cancer is local escape from the confines of the mammary duct into the adjacent tissue stroma. In the normal mammary gland, epithelial ductal and alveolar structures are surrounded by a contractile myoepithelial cell layer which facilitates milk expulsion during lactation [3]. The mammary myoepithelial cells are also required for normal mammary gland development, as they influence epithelial cell polarity, ductal branching and milk production [3]. A hallmark of progression from DCIS to invasive cancer is physical breach of this myoepithelial cell layer. With respect to tumor progression, recent studies suggest that myoepithelial cells play an active role in tumor suppression by secreting protease inhibitors, downregulating matrix metalloproteinases [4-8], and producing tumor suppressive proteins such as maspin, p63, Wilm's tumor 1, p73 and 14-3-3Sigma [7-12]. According to this theory, the tumor suppressive nature of myoepithelial cells is lost with tumor progression, facilitating the transition from a pre-invasive state to invasive cancer [6, 7]. Another hypothesis posits that tumor cells and/or accumulation of other reactive non-epithelial cells, such as fibroblasts or immune cells, lead to the breakdown of the protective myoepithelial barrier and "escape" of

tumor epithelial cells [6, 7, 13]. Studies have reported that DCIS-associated myoepithelial cells are phenotypically different than normal myoepithelial cells [5, 14]; and tumor cells adjacent to focal myoepithelial cell layer disruptions can display distinct phenotypes including estrogen receptor (ER) negativity, genetic instabilities, increased expression of invasion-related genes, and aberrant e-cadherin expression [13, 15, 16]. Overall, these data support the notion that interplay between tumor cells and the myoepithelial cells contribute to loss of the myoepithelial cell layer that is critical for the transition to invasive disease.

Studying tumor progression in the mammary gland is further complicated by the fact that the gland undergoes dramatic functional and compositional changes with hormone exposure, and hormone-driven events such as menarche, menstrual cycling, pregnancy and lactation all impact breast cancer risk in women [17-21]. Evidence that the host reproductive state influences breast cancer progression has been obtained in pre-clinical studies. For example, using 3D culture and mammary fat pad models, we have shown that postpartum involution, a period of active mammary gland tissue remodeling following weaning, creates a wound-healing like microenvironment that is permissive to tumor growth and metastatic spread [22-24]. For the study of early stage DCIS progression, a model that permits assessment of host physiology is lacking.

Tumorigenic potential of human mammary epithelial tumor cell lines is primarily evaluated by injecting cells into the mammary fat pads (MFP) of immunocompromised mice [25]. While the MFP is the correct organ host for invasive breast cancer, it is not the correct anatomical location for early stage, pre-invasive tumors. With respect to tumor progression, MFP models bypass the requirement for tumor cells to exit from the location of their initiation, i.e., the mammary ducts. Alternatively, transgenic models offer correct anatomical location of breast

cancer and display tumor progression from initiation to hyperplasia to invasive stages, but since all epithelial cells contain the active oncogene, these models do not replicate transformation as a rare event. An intraductal approach in the absence of surgery offers a key advantage in that cells are directly placed in the correct anatomical location for tumor initiation, which permits modeling of the natural history of disease progression in the background of normal mammary ducts. Further, this approach permits co-evolution of tumor progression with myoepithelial cell changes as well as stromal changes with minimal wound healing or pro-inflammatory induction.

MATERIALS AND METHODS

Animals

5-week old female SCID mice were obtained from Taconic (Hudson, NY) and maintained in the Center for Laboratory Animal Care at the University of Colorado Anschutz Medical Campus. Females were bred at 8-9 weeks of age as previously described [26] and weaned between 10-14 days post-parturition to initiate involution on the same calendar date. Age-matched nulliparous female mice were used as controls. Intraductal tumor inections were performed as described below. Mammary tissue was excised from animals euthanized by carbon dioxide exposure followed by cervical dislocation. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus (protocol #72110(07)1E).

Cell Culture

MCF10ADCIS.com cells and GFP-labeled MCF10ADCIS.com cells (a generous gift from Kornelia Polyak) were cultured as previously described [5] and resuspended in PBS immediately prior to injection. Cells were used between passages 8 – 22, as passages later than 22 have been shown to display a more invasive phenotype [27].

Intraductal Injections for Tumor Studies

3-10µl of 50,000 MCF10ADCIS.com cells were intraductally injected into anesthetized mice using a previously described intraductal delivery method developed for viral delivery [28, 29]. Briefly, the end of a 25µl Wiretrol II disposable glass micropipette (no. 5-000-2050; Drummond Scientific Company, Broomall, PA) was drawn and fire-polished into a fine tip of 60-75µm. Sterile cell solution was loaded into the micropipette with a stainless steel plunger. Using a micromanipulator, the tip was gently inserted directly into the teat canal and cells slowly ejected

into the lumens of the left third thoracic and both fourth inguinal mammary glands of mice (n = 3-4 injected glands /mouse). To study the effect of postpartum involution on tumor growth, cells were intradutally injected into mammary glands of involution group mice 2 days post-weaning (n = 2-3 injected glands/mouse) or age-matched nulliparous control group mice (n = 2-3 injected glands/mouse). The third right thoracic mammary glands served as non-injected controls for all injected mice. Images depicting the intraductal injection technique were captured using a Canon PowerShot A620 camera with 4X optical zoom.

Determination of Mammary Ductal Epithelium Permeability

Epithelial tight junction permeability as a function of injection volume was assessed as previously described [29, 30]. Briefly, on the day of sacrifice, 2 μCi of [^{14}C]-sucrose (Amersham, Buckinghamshire, United Kingdom) was lyophilized and dissolved in sterile Trypan blue. Either 5μl or 10μl of this solution was intraductally injected into the 4th inguinal mammary glands of each mouse (n = 2) under anesthesia. 10μl blood samples were taken from the tail vein 5 min after each injection, and the amount of ^{14}C present was determined by liquid scintillation counting.

Immunohistochemistry, Imaging, and Quantification

Excised mouse mammary glands were fixed in 10% NBF, paraffin embedded , and prepared for hemotoxylin and eosin staining as previously described [26]. Briefly, entire histological sections were scanned as described and total tumor number and area were quantified using Aperio Spectrum software (Aperio Techologies, Vista, CA). For immunohistochemistry, 4μm sections of paraffin embedded mouse mammary gland tissue were pretreated with Dako TRS Antigen Retriveal Solution (TRS) or Dako EDTA Antigen Retrieval Solution (EDTA). Research using de-identified human breast tissue as positive IHC controls was conducted under a protocol

deemed exempt from subject consent as approved by the Colorado Multiple Institution Review Board (COMIRB) and tissues were acquired as previously reported [26]. The following antibodies, antigen retrievals, and antibody dilutions were used: mouse anti-human estrogen receptor (ER) (EDTA, 1:100; Leica Microsystems, Inc., Buffalo Grove, IL), mouse anti-human progesterone receptor (PR) (TRS, 1:400; Dako, Carpinteria, CA), rabbit anti-human HER2 (TRS, 1:500; Dako), mouse anti-human e-cadherin (TRS, 1:500; BD Biosciences, San Jose, CA), mouse anti-human p63 (EDTA, 1:200; BioCare Medical, Concord, CA), mouse anti-human cytokeratin 5 (CK5) (EDTA, 1:50; BioCare Medical, Concord, CA), rabbit anti-human calponin (EDTA, 1:800; Abcam, Cambridge, MA), mouse anti-human smooth muscle actin (SMA) (EDTA, 1:200; Dako). ER positivity was assessed according to the Allred scoring method [31]. HER2 positivity was determined using the FDA approved Hercep test for the Dako Autostainer. For myoepithelial cell layer integrity quantitation, tumor associated myoepithelial cell expression of SMA, calponin, and p63 was obtained using serial IHC sections. Data is presented as precent of tumors/group suurounded by ≥50% positivity (arbitrary cutoff), with 25 tumors per group analyzed.

Fluorescent In Situ Hybridization (FISH) Analysis

FISH analyses were performed using probes for human and mouse Cot-1 DNA, as previously described [32], by the University of Colorado Cancer Center Cytogenetics Core.

Statistical Analysis

Statistical significance was determined by Student's t-test where applicable.

RESULTS AND DISCUSSION

Attributes of the Intraductal Model

We focus our analysis using MCF10A DCIS.com cells as a representative human breast cancer cell line capable of forming early stage DCIS-like lesions *in vivo* [5, 27, 33]. Derived from the MCF10AT cell line, these cells meet the criteria for a model of human breast cancer progression as they display histologic progression from hyperplasia to DCIS to invasive carcinoma in subcutaneous, mammary fat pad and intraductal injection models [5, 27, 33, 34]. Here, MCF10A DCIS.com cells were intraductally injected into an intact teat to minimize inflammation induced by surgical manipulations (Figure 1). In nulliparous hosts, the tumor take rate was 65%. Given that progression to invasive disease requires disruption of epithelial junctional integrity as well as loss of the normally protective myoepithelial cell layer, it is imperative that ductal integrity not be compromised during the intraductal procedure. To address this issue, we determined the optimal injection volume to ensure that the technical aspects of intraductal delivery did not disrupt integrity of the mouse mammary epithelial junctional complexes or the myoepithelial cell layer. We employed serial IHC analysis to confirm the presence of GFP-labeled MCF10ADCIS.com cells [24] injected at 2.5 µl, 5.0 µl. or 10 µl volumes (Figure 2A, a,d,g) and the effect of the different injection volumes 24 hours post-injection on the integrity of the epithelial and myoepithelial cell layers using e-cadherin (Figure 2A, b,e,h) and smooth muscle actin (Figure 2A, c,f,i), respectively. Although there was no evidence of GFP positive tumor cells within the mammary stroma at any of the volumes of cells injected (Figure 2A, d,g), we found small areas of epithelial and myoepithelial breakdown (Figure 2A, a-c) at an injection volume of 2.5 µl. These disruptions were not seen at 5µl or 10 µl injection volumes (Figure 2A, d-i). One possible explanation is that delivery of cells in volumes

of less than 5 µl may not allow sufficient dispersion of tumor cells within the ducts, leading to local disruption.

Whether or not junctional integrity is compromised in the nulliparous mammary gland via intraductal injection cannot be fully addressed using IHC. Previous studies have analyzed tight junction permeability of mammary epithelial cells during secretory activation by intraductally injecting ^{14}C-sucrose and measuring its presence in the blood [30, 35]. We assessed ^{14}C-sucrose permeability in nulliparous glands and found that while injected dye volumes of 5 µl do not appear to compromise ductal integrity, volumes of 10 µl or greater may disrupt junctional complexes (Figure 2B). Therefore, for subsequent studies, we elected a 5 µl volume of tumor cells as optimal volume for injecting nulliparous mammary glands.

Our histological data of MCF10ADCIS.com tumors corroborate and extend previous observations by showing intraductal DCIS lesions that display characteristics of the main human DCIS subtypes: solid, cribriform, pseudo-papillary, comedo, and apocrine (Figure 3A, a-e). Further, these tumor cells have the ability to locally invade into adjacent stroma (Figure 3A, f). The distribution of lesions with hyperplasia, DCIS or invasive characteristics (tumor stage) at 4 weeks post injection was 28%, 52%, and 20%, respectively (Figure 3B). Evidence that this phenotypic distribution is due to disease progression is strongly suggested by the observation that at one week post-injection, very few invasive lesions are observed (Figure 3C), whereas later time points more numerous invasive stage lesions are observed (Figure 3A, f).

Unique to the intraductal delivery model, MCF10ADCIS.com cells are observed to form epithelial intraductal hyperplasias (Figure 3D, a) and incorporate into the normal mouse ductal epithelium (Figure 3D, b) as detected by fluorescent in situ hybridization (FISH) analysis with probes specific for human (red) and mouse (green) COT-1 DNA. In agreement with data

obtained from other intraductal models [33], lesions formed from MCF10ADCIS.com cells utilize the endogenous mouse myoepithelial layer (arrows , Figure 3D, a,b), and do not generate a human cell derived myoepithelial cell layer as observed in the subcutaneous and fat pad models [5, 27, 36]. We next evaluated whether these human tumor cells form E-cadherin-based adherens junctions with neighboring mouse epithelial cells within the mammary duct. Formation of adherens junctions would confirm that very early stage tumor lesions develop in the intraductal model, and further, adherens junction presence would permit the evaluation of their disruption with disease progression [37]. Our data indicate that MCF10ADCIS.com cells form junctional complexes with normal mouse epithelial cells, as well as with neighboring tumor cells, albeit at a lower staining intensity (Figure 3E, a). Confirmation that human tumor cells and normal mouse mammary epithelial cells within the duct form adhesion junctional complexes was obtained by serial FISH analysis (Figure 3E, b), and demonstrate the relevance of this model for the study of tumor cell escape/invasion out of the mammary duct.

Our intraductal approach offers an advantage to study host effects on various subtypes of breast cancer. Xenograft tumors formed by MCF10ADCIS.com cells have been previously characterized as estrogen receptor (ER), progesterone receptor (PR) and HER2 negative and positive for the basal phenotype marker, cytokeratin 5 (CK5) [24, 33]. Prior to injection, MCF10ADCIS.com cells express cytokeratin 5 (Figure 4, a), but not ER (Figure 4, b), PR (Figure 4, c) or HER2 (Figure 4, d). Surprisingly, in contrast to a previously described intraductal xenograft model [33] , MCF10ADCIS.com cells injected intraductally re-expressed ER in a subset of cells (Figure 4, f). HER2 status was indeterminate by IHC (Figure 4, h). All staining was confirmed by IHC analysis on respective positive controls (Figure 4, i-l). Although the question of why ER is preferentially re-expressed in MCF10ADCIS.com cells when

intraductally injected is beyond the scope of this present work, it sheds light on the unique nature of this model for the study of host-tumor interactions at early stages of disease.

Characterization of the Myoepithelial Cell Barrier with DCIS Progression

Using a panel of myoepithelial specific markers, SMA, calponin [38-40], and p63 [5, 9, 41], we assessed DCIS lesions to determine if loss of these markers occurs with disease progression. SMA, calponin and p63 expression pattern in normal, non-tumor bearing ducts is evident from staining of mammary tissue (Figure 5A, a, d, g). The myoepithelial cell layer surrounding some DCIS lesions have high levels of all three myoepithelial cell markers, indicating a non-compromised myoepithelial cell layer (Figure 5A, b, e, h). However, many DCIS lesions are surrounded by a potentially compromised myoepithelial cell layer, as evidenced by lower expression levels of these markers (Figure 5A, c, f, i). Quantification of these data show that the myoepithelial cell layer surrounding DCIS-lesions have lower expression of SMA, calponin, and p63 compared to normal ducts (Figure 5B). Additionally, p63 appears to be lost earlier than SMA or calponin, as many myoepithelial layers surrounding DCIS-lesions were SMA and calponin positive, but p63 negative (data not shown). Further, loss of p63 in the myoepithelial cell layer correlated with gain of p63 in the tumor cells (compare 5A h and i). These later data suggest that myoepithelial p63 expression is differentially regulated between myoepithelial cells and tumor cells at the transition to invasiveness. Cumulatively, these studies demonstrate the ability to investigate myoepithelial cell changes associated with DCIS progression using this intraductal model.

Influence of Physiological Reproductive State on Tumor Progression

One strength of an intraductal model in the absence of surgical manipulation is that it permits the non-confounded investigation of host factors on tumor progression. In a xenograft

mammary fat pad model, we have previously demonstrated that the postpartum involuting mammary gland is characterized by a wound-healing like microenvironment that is tumor promotional [23, 24, 26, 32, 42]. An unanswered question is whether the postpartum mammary gland would be similarly tumor promotional for lesions confined within the mammary ducts. Our analysis shows that more tumors form in the involution group (Figures 6A and 6C, b) and these tumors are larger in size (Figures 6B and 6C, b) compared to their respective nulliparous controls (Figure 6C, a). Thus, our intraductal model allows us to assess the effects of reproductive state on DCIS progression to invasion, and demonstrates for the first time, the ability of postpartum involution to influence progression of tumor cells confined within the ducts.

CONCLUSIONS

Our preclinical intraductal model of human breast cancer provides a rigorous approach to study breast cancer progression from early-stage ductal hyperplasia to invasion. An intraductal approach recently described offers a major advancement over MFP models by depositing cells in a large volume (200µl) within the surgically exposed lactiferous duct, which permits lymph node metastasis not observed in MFP models [43]. However, our data using epithelial cell tight junction disruption as a marker for loss of epithelial integrity demonstrate that the normal ductal networks within the mammary gland are likely disrupted with volumes exceeding 10µl, suggesting that the "large volume" intraductal method prohibits the study of early-stage tumor cell incorporation into the ductal lining of the host mammary gland as well as changes in the myoepithelial cell layer with tumor progression. Another intraductal model injects a small volume of tumor cells unlikely to compromise duct integrity, but surgical manipulations are used to cleave the teat and expose the main lactiferous duct [33]. These surgical procedures may induce localized wound-healing and inflammatory programs, which have been shown to be tumor promotional in other contexts [44-47]. In this report, we describe an intraductal model that minimizes wound-healing programs and inflammation, and which permits the evaluation of subtle physiologic changes in tissue remodeling without the confounder of wound-healing programs associated with surgical manipulation or physical duct disruption. A limitation to the method described here, and all other human tumor models, is the requirement for immunodeficient animals. The application of our intraductal model utilizing isogenic mammary cancer cell lines that reflect the biologic subtypes of human breast cancer would permit investigation of DCIS progression in an immune competent host.

Surprisingly, ER is re-expressed when MCF10ADCIS.com cells are intraductally injected into mouse mammary glands (Figure 4). This inherent plasticity of human breast cancer cell lines can cause a conundrum for developing intraductal models to study the influence of breast cancer biologic subtype on progression from DCIS to invasive cancer. However, additional studies with multiple cell lines are to needed to determine if the re-expression of these markers is cell line specific or more generalizable. Nevertheless, human versus mouse myoepithelial derivation between xenograft and intraductal models may influence stem/progenitor characteristics of MCF10ADCIS.com cells. For example, in vitro studies characterize MCF10ADCIS.com cells as bipotential progenitors, and xenograft studies show that these cells differentiate into both luminal and myoepithelial cells when injected subcutaneously or into the MFP [5, 27, 36]. Our data corroborate recently reported studies showing that MCF10ADCIS.com cells delivered intraductally utilize mouse myoepithelia [33]. In the intraductal model, it is possible that ER+ MCFDCIS10A.com cells may derive from progenitors that are influenced by ER+ mouse luminal cells and or stem cell niches within the host mammary ducts [48]. Further work is needed to explore these possibilities.

Our model also permits a rigorous evaluation of the effects of DCIS progression on molecular changes in the myoepithelium, which serves as a barrier to invasive cancer. Our data suggest that this protective layer is compromised during early stages of tumor progression. We focused our initial studies on three myoepithelial cell markers: SMA, calponin, and p63, loss of which is commonly used to identify DCIS progression to invasive disease [49-51]. SMA positivity surrounding tumors remains relatively stable (Figure 5, a-c), suggesting that the actin biostructure detected with anti-SMA antibody may be a myoepithelial barrier lost later DCIS progression. Calponin, an important actin cytoskeleton regulator [52-54] and tumor suppressor in

human leiomyosarcoma [54] also appears to remain stable surrounding DCIS lesions (Figure 5, d-f). We show that p63 expression appears to be lost prior to that of SMA and calponin, and may indicate compromised barrier function in myoepithelial cells. However, intratumoral p63 is also evident in DCIS lesions formed by MCF10ADCIS.com cells, indicating acquisition of basal-like attributes with progression (Figure 5a, i). Upregulation of p63 in DCIS lesions formed by MCF10ADCIS.com cells has been previously shown to be near regions of compromised myoepithelium [36]. These data suggest the use of p63 as a myoepithelial marker may not give an accurate analysis of myoepithelial integrity from tumors with a basal phenotype.

Further, we demonstrate proof-of-principal that host physiology can influence DCIS progression, identifying this model as relevant for addressing key questions such as influence of host reproductive status on breast cancer progression. Emerging work from our laboratory in multiple animal models of postpartum breast cancer shows that the microenvironment of the involuting gland is highly supportive of tumor cell dissemination and metastasis [23, 24, 55]. The development of this new intraductal model will permit a more physiologically relevant investigation into the interactions between reproduction state and DCIS progression. Future studies include further evaluation of the effect of host reproductive state on tumor suppressive related myoepithelial cell markers.

In summary, our preclinical intraductal model of human breast cancer provides a rigorous approach to studying tumor progression from early stage intraductal hyperplasia to invasion. This model is particularly suited to studying host effects on DCIS progression. Since occult tumors in women develop within ducts, we propose that this teat injection model will facilitate research of early disease progression, a requisite for research focused on breast cancer prevention and inhibition of local invasion.

ABBREVIATIONS:

DCIS	ductal carcinoma in situ
ER	estrogen receptor
MFP	mammary fat pad
FISH	fluorescent in situ hybridization
SMA	smooth muscle actin
CK5	cytokeratin 5

COMPETING INTERESTS: We have no competing interests to declare.

AUTHORS' CONTRIBUTIONS

TDR designed and performed all animal experiments, performed the intraductal injections, IHC, ^{14}C-sucrose experiments, interpreted and analyzed data, and prepared the manuscript. PS supervised the project, designed experiments, interpreted results, and made substantial contributions to manuscript preparation.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Peggy Neville (University of Colorado Department of Obstetrics and Gynecology) for support and guidance; Drs. Virginia Borges (University of Colorado Division of Medical Oncology), Carol Sartorius (Department of Pathology), and Jennifer Richer (Department of Pathology) for critical manuscript review; Dr. Marileila Garcia (University of Colorado Cancer Center Cytogenetics Core and Division of Medical Oncology) for FISH analysis; UCD Prostate Cancer Research Laboratory for image scanning; Dr. Sonali Jindal for

contribution to data analysis; Patricia Bell and Samiat Agunbiade for extensive IHC preparation; and Jaime Fornetti for critical manuscript review and intraductal technique image acquisition. This work was supported by an NIH T32 Training Grant “Training in Pharmacology of Antineoplastic Agents” (Division of Medical Oncology, University of Colorado AMC); University of Colorado AMC Thorkildsen Research Fellowship; UNCF Merck Postdoctoral Science Research Fellowship; and Department of Defense Postdoctoral Award BC096776 to TDR and Komen Foundation KG090629 and Department of Defense Award BC060531 to PS.

REFERENCES

1. Wellings SR, Jensen HM: **On the origin and progression of ductal carcinoma in the human breast**. *Journal of the National Cancer Institute* 1973, **50**(5):1111-1118.
2. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT *et al*: **Gene expression profiling predicts clinical outcome of breast cancer**. *Nature* 2002, **415**(6871):530-536.
3. Sopel M: **The myoepithelial cell: its role in normal mammary glands and breast cancer**. *Folia Morphol (Warsz)* 2010, **69**(1):1-14.
4. Barsky SH, Karlin NJ: **Myoepithelial cells: autocrine and paracrine suppressors of breast cancer progression**. *J Mammary Gland Biol Neoplasia* 2005, **10**(3):249-260.
5. Hu M, Yao J, Carroll DK, Weremowicz S, Chen H, Carrasco D, Richardson A, Violette S, Nikolskaya T, Nikolsky Y *et al*: **Regulation of in situ to invasive breast carcinoma transition**. *Cancer Cell* 2008, **13**(5):394-406.
6. Polyak K, Hu M: **Do myoepithelial cells hold the key for breast tumor progression?** *J Mammary Gland Biol Neoplasia* 2005, **10**(3):231-247.
7. Man YG, Sang QX: **The significance of focal myoepithelial cell layer disruptions in human breast tumor invasion: a paradigm shift from the "protease-centered" hypothesis**. *Exp Cell Res* 2004, **301**(2):103-118.
8. Sternlicht MD, Barsky SH: **The myoepithelial defense: a host defense against cancer**. *Med Hypotheses* 1997, **48**(1):37-46.
9. Barbareschi M, Pecciarini L, Cangi MG, Macri E, Rizzo A, Viale G, Doglioni C: **p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast**. *Am J Surg Pathol* 2001, **25**(8):1054-1060.
10. Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, Rafidi K, Seftor E, Sager R: **Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells**. *Science* 1994, **263**(5146):526-529.
11. Li JH, Man YG: **Dual usages of single Wilms' tumor 1 immunohistochemistry in evaluation of breast tumors: a preliminary study of 30 cases**. *Cancer Biomark* 2009, **5**(3):109-116.
12. Simpson PT, Gale T, Reis-Filho JS, Jones C, Parry S, Steele D, Cossu A, Budroni M, Palmieri G, Lakhani SR: **Distribution and significance of 14-3-3sigma, a novel myoepithelial marker, in normal, benign, and malignant breast tissue**. *J Pathol* 2004, **202**(3):274-285.
13. Man YG, Tai L, Barner R, Vang R, Saenger JS, Shekitka KM, Bratthauer GL, Wheeler DT, Liang CY, Vinh TN *et al*: **Cell clusters overlying focally disrupted mammary myoepithelial cell layers and adjacent cells within the same duct display different immunohistochemical and genetic features: implications for tumor progression and invasion**. *Breast Cancer Res* 2003, **5**(6):R231-241.
14. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A *et al*: **Molecular characterization of the tumor microenvironment in breast cancer**. *Cancer Cell* 2004, **6**(1):17-32.
15. Zhang X, Hashemi SS, Yousefi M, Gao C, Sheng J, Ni J, Wang W, Mason J, Man YG: **Atypical E-cadherin expression in cell clusters overlying focally disrupted**

mammary myoepithelial cell layers: implications for tumor cell motility and invasion. *Pathol Res Pract* 2009, **205**(6):375-385.
16. Zhang X, Hashemi SS, Yousefi M, Ni J, Wang Q, Gao L, Gong P, Gao C, Sheng J, Mason J *et al*: **Aberrant c-erbB2 expression in cell clusters overlying focally disrupted breast myoepithelial cell layers: a trigger or sign for emergence of more aggressive cell clones?** *Int J Biol Sci* 2008, **4**(5):259-269.
17. Chie WC, Hsieh C, Newcomb PA, Longnecker MP, Mittendorf R, Greenberg ER, Clapp RW, Burke KP, Titus-Ernstoff L, Trentham-Dietz A *et al*: **Age at any full-term pregnancy and breast cancer risk**. *Am J Epidemiol* 2000, **151**(7):715-722.
18. Stuebe AM, Willett WC, Xue F, Michels KB: **Lactation and incidence of premenopausal breast cancer: a longitudinal study**. *Arch Intern Med* 2009, **169**(15):1364-1371.
19. Palmer JR, Boggs DA, Wise LA, Ambrosone CB, Adams-Campbell LL, Rosenberg L: **Parity and lactation in relation to estrogen receptor negative breast cancer in African American women**. *Cancer Epidemiol Biomarkers Prev* 2011, **20**(9):1883-1891.
20. Rosner B, Colditz GA: **Nurses' health study: log-incidence mathematical model of breast cancer incidence**. *Journal of the National Cancer Institute* 1996, **88**(6):359-364.
21. Pike MC: **Age-related factors in cancers of the breast, ovary, and endometrium**. *J Chronic Dis* 1987, **40 Suppl 2**:59S-69S.
22. Schedin P, Elias A: **Multistep tumorigenesis and the microenvironment**. *Breast Cancer Res* 2004, **6**(2):93-101.
23. Schedin P: **Pregnancy-associated breast cancer and metastasis**. *Nat Rev Cancer* 2006, **6**(4):281-291.
24. Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan AC, Schedin P: **Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2**. *Nat Med* 2011, **17**(9):1109-1115.
25. Price JE, Polyzos A, Zhang RD, Daniels LM: **Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice**. *Cancer Res* 1990, **50**(3):717-721.
26. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man YG, Borges V, Schedin P: **Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species**. *The American journal of pathology* 2010, **176**(3):1241-1255.
27. Miller FR, Santner SJ, Tait L, Dawson PJ: **MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ**. *Journal of the National Cancer Institute* 2000, **92**(14):1185-1186.
28. Nguyen D-AD, Beeman NG, Lewis MT, Schaack J, Neville MC: **Intraductal injection into the mouse mammary gland.** In: *Methods in Mammary Gland Biology and Breast Cancer Research.* Edited by Ip MM, Asch BB. N.Y.: Kluwer Academic/Plenum; 2000: 259-270.
29. Russell TD, Fischer A, Beeman NE, Freed EF, Neville MC, Schaack J: **Transduction of the mammary epithelium with adenovirus vectors in vivo**. 2003, **77**(10):5801-5809.
30. Nguyen D-AD, Parlow AF, Neville MC: **Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation**. *Journal of Endocrinology* 2001, **170**(2):347-356.

31. Qureshi A, Pervez S: **Allred scoring for ER reporting and it's impact in clearly distinguishing ER negative from ER positive breast cancers**. *J Pak Med Assoc* 2010, **60**(5):350-353.
32. McDaniel SM, Rumer KK, Biroc SL, Metz RP, Singh M, Porter W, Schedin P: **Remodeling of the mammary microenvironment after lactation promotes breast tumor cell metastasis**. *The American journal of pathology* 2006, **168**(2):608-620.
33. Behbod F, Kittrell FS, Lamarca H, Edwards D, Kerbawy S, Heestand JC, Young E, Mukhopadhyay P, Yeh HW, Allred DC *et al*: **An intra-ductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ**. *Breast Cancer Res* 2009, **11**(5):R66.
34. Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ, Heppner GH: **Xenograft model of progressive human proliferative breast disease**. *Journal of the National Cancer Institute* 1993, **85**(21):1725-1732.
35. Nguyen D-AD, Neville MC: **Tight junction regulation in the mammary gland**. 1998, **3**(3):233-246.
36. Shekhar MP, Tait L, Pauley RJ, Wu GS, Santner SJ, Nangia-Makker P, Shekhar V, Nassar H, Visscher DW, Heppner GH *et al*: **Comedo-ductal carcinoma in situ: A paradoxical role for programmed cell death**. *Cancer Biol Ther* 2008, **7**(11):1774-1782.
37. Prasad CP, Rath G, Mathur S, Bhatnagar D, Parshad R, Ralhan R: **Expression analysis of E-cadherin, Slug and GSK3beta in invasive ductal carcinoma of breast**. *BMC Cancer* 2009, **9**:325.
38. Deugnier MA, Moiseyeva EP, Thiery JP, Glukhova M: **Myoepithelial cell differentiation in the developing mammary gland: progressive acquisition of smooth muscle phenotype**. *Dev Dyn* 1995, **204**(2):107-117.
39. Xu Z, Wang W, Deng CX, Man YG: **Aberrant p63 and WT-1 expression in myoepithelial cells of pregnancy-associated breast cancer: implications for tumor aggressiveness and invasiveness**. *Int J Biol Sci* 2009, **5**(1):82-96.
40. Zhang RR, Man YG, Vang R, Saenger JS, Barner R, Wheeler DT, Liang CY, Vinh TN, Bratthauer GL: **A subset of morphologically distinct mammary myoepithelial cells lacks corresponding immunophenotypic markers**. *Breast Cancer Res* 2003, **5**(5):R151-156.
41. Reis-Filho JS, Schmitt FC: **Taking advantage of basic research: p63 is a reliable myoepithelial and stem cell marker**. *Adv Anat Pathol* 2002, **9**(5):280-289.
42. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V: **Microenvironment of the involuting mammary gland mediates mammary cancer progression**. *J Mammary Gland Biol Neoplasia* 2007, **12**(1):71-82.
43. Harrell JC, Dye WW, Harvell DM, Pinto M, Jedlicka P, Sartorius CA, Horwitz KB: **Estrogen insensitivity in a model of estrogen receptor positive breast cancer lymph node metastasis**. *Cancer Res* 2007, **67**(21):10582-10591.
44. Coussens LM, Werb Z: **Inflammation and cancer**. *Nature* 2002, **420**(6917):860-867.
45. Schafer M, Werner S: **Cancer as an overhealing wound: an old hypothesis revisited**. *Nat Rev Mol Cell Biol* 2008, **9**(8):628-638.
46. Martins-Green M, Boudreau N, Bissell MJ: **Inflammation is responsible for the development of wound-induced tumors in chickens infected with Rous sarcoma virus**. *Cancer Res* 1994, **54**(16):4334-4341.

47. Stuelten CH, Barbul A, Busch JI, Sutton E, Katz R, Sato M, Wakefield LM, Roberts AB, Niederhuber JE: **Acute wounds accelerate tumorigenesis by a T cell-dependent mechanism**. *Cancer Res* 2008, **68**(18):7278-7282.
48. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ: **Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland**. *J Cell Biol* 2007, **176**(1):19-26.
49. Hilson JB, Schnitt SJ, Collins LC: **Phenotypic alterations in ductal carcinoma in situ-associated myoepithelial cells: biologic and diagnostic implications**. *Am J Surg Pathol* 2009, **33**(2):227-232.
50. Bhargava R, Dabbs DJ: **Use of immunohistochemistry in diagnosis of breast epithelial lesions**. *Adv Anat Pathol* 2007, **14**(2):93-107.
51. Lerwill MF: **Current practical applications of diagnostic immunohistochemistry in breast pathology**. *Am J Surg Pathol* 2004, **28**(8):1076-1091.
52. Mezgueldi M, Mendre C, Calas B, Kassab R, Fattoum A: **Characterization of the regulatory domain of gizzard calponin. Interactions of the 145-163 region with F-actin, calcium-binding proteins, and tropomyosin**. *J Biol Chem* 1995, **270**(15):8867-8876.
53. North AJ, Gimona M, Cross RA, Small JV: **Calponin is localised in both the contractile apparatus and the cytoskeleton of smooth muscle cells**. *Journal of cell science* 1994, **107 (Pt 3)**:437-444.
54. Rozenblum GT, Gimona M: **Calponins: adaptable modular regulators of the actin cytoskeleton**. *Int J Biochem Cell Biol* 2008, **40**(10):1990-1995.
55. O'Brien J, Schedin P: **Macrophages in breast cancer: do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer?** *J Mammary Gland Biol Neoplasia* 2009, **14**(2):145-157.

FIGURE LEGENDS

Figure 1. Intraductal injection method. A small area surrounding the teat (arrow) was aseptically cleared of fur (A). A fabricated glass micropipette with 60-75µm tip diameter was then positioned near the intact teat (B) and carefully guided into the teat (C, arrow). Image in C was taken through the eyepiece of a dissecting scope at 4X magnification.

Figure 2. Ductal and myoepithelial cell layer integrity is not compromised by intraductal injection of 5.0 µl volumes. A. GFP-labeled MCF10ADCIS.com cells were injected at volumes of 2.5 µl (panels a-c), 5.0 µl (panels d-f), and 10 µl (panels g-i) to assess epithelial junctional complex and myoepithelial cell layer disruption. Serial sections were stained with GFP to confirm the presence of human tumor cells (brown, panels a, d, g); e-cadherin (eCAD) to assess ductal epithelia junctional complex disruption (brown, panels b, e, h); and smooth muscle actin (SMA) to assess myoepithelial cell layer disruption (brown, panels c, f, and i). Insets in panels a-c are enlarged to show detail of compromised ductal areas. Images scanned using Aperio software. Digital resolution = 0.25µ/pixel. Scale bar = 100um. B. Ductal epithelia tight junction permeability as a function of volume was assessed by intraductally injecting mice with the stated doses of Trypan blue dye mixed with [^{14}C]-sucrose in nulliparous mice. Each bar represents the average of [^{14}C] counts per minute (CPM) measured in the blood. n = 2/group.

Figure 3. DCIS characteristics of MCF10ADCIS.com cells. A. Hemotoxylin and eosin (H&E) analysis of nulliparous mouse mammary glands injected with MCF10ADCIS.com tumor cells via intraductal method. Tumors formed from MCFDCIS10A.com cells display the different

characteristics of human DCIS (solid – a; cribriform – b; pseudo papillary – c; comedo – d; apocrine – e), and progress to locally invasive disease (f). Images scanned using Aperio software; digital resolution = 0.25µ/pixel. Scale bar = 80µm. B. Distribution of tumor stage in nulliparous mammary glands intraductally injected with MCF10ADCIS.com cells. C. Representative H&E image of a non-invasive MCF10ADCIS.com tumor bolus (arrow) formed 8 days post injection. Image scanned using Aperio software; digital resolution = 0.25µ/pixelScale bar = 60µm. D. Fluorescent in situ hybridization (FISH) analysis for human (red) and mouse (green) COT-1 DNA reveals tumor progression from hyperplastic alveolar nodules (a) to DCIS (b) with incorporation of human tumor cells into normal mouse mammary ducts (circle). Arrows indicate myoepithelial cells. Magnification = 40X. Scale bars = 40µm. E. Serial analysis of epithelial cell junctional integrity. Left panel: IHC analysis of the cytoplasmic domain of E-cadherin (brown). Right panel: FISH analysis of human (red) and mouse (green) COT-1 DNA. E-cadherin-based junctional complexes form between MCF10ADCIS.com cells (white arrows) and between MCF10ADCIS.com cells and mouse ductal epithelial cells (arrowheads). Magnification = 100X. Scale bar = 10µm.

Figure 4. MCF10ADCIS.com cells re-express estrogen receptor in vivo. Serial analysis of MCF10ADCIS.com cells prior to intraductal injection demonstrating triple negative characteristic of these cells (panels a-d) and ER+ tumors formed 4 weeks post-intraductal injection (panel f). Insets in panels f-h are enlarged to show detail of CK5 (e), ER (f), PR (g), and HER2 (h) staining. Positive normal mammary gland (i) and breast tumor (j, k, l) controls are stained for CK5 (i), ER (j), PR (k), and HER2 (l). Images scanned using Aperio software. Digital resolution = 0.25µ/pixel. Scale bar for panels a-d = 50µm. Scale bar for panels e-l = 50µm.

Figure 5. IHC Analysis Suggests Progressive Loss of Myoepithelial Cell Differentiation Markers. A. Serial IHC analysis of myoepithelial cell markers SMA (brown, panels a-c), calponin (brown, panels d-f), and p63 (brown, panels g-i). Insets in panels a, d, and g are enlarged to show detail of normal mouse ductal structures. Some tumors show no apparent loss of myoepithelial cell layer markers (panels b, e, h) while other tumors display loss of these markers (panels c, f, i). Gain of p63 by tumor cells is also apparent (panels h, i). Images scanned using Aperio software. Digital resolution = 0.25μ/pixel. Scale bar = 100 μm. B. Quantification of percentage of tumors surrounded by ≥50% (arbitrary cutoff) myoepithelial cell layer marker, compared to the percentage of normal, non-tumor bearing ductal structures surrounded by ≥50% myoepithelial cell layer marker.

Figure 6. Use of the intraductal approach to analyze physiological impact of reproductive state on tumor progression. Tumor bearing mammary glands from nulliparous and involuting mice were assessed for number and size of tumors using Aperio digital software. A. Total numbers of tumors from each group. The involution group (n = 2) had more tumors than their respective nulliparous control group (n = 1). B. Tumors from the involution group were larger than their respective nulliparous control. Error bars = SEM. C. Representative H&E stained images from nulliparous and involution group tumor bearing mammary glands. Images scanned and tumors measured using Aperio software. Digital resolution = 0.25μ/pixel. Scale bar = 70 μm.

Figure 1

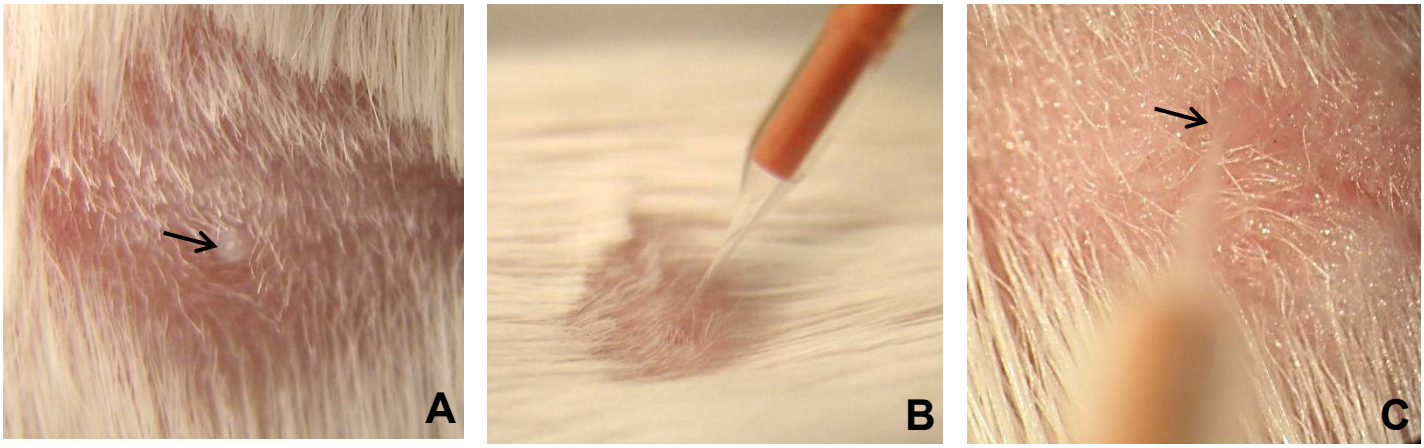


Figure 2

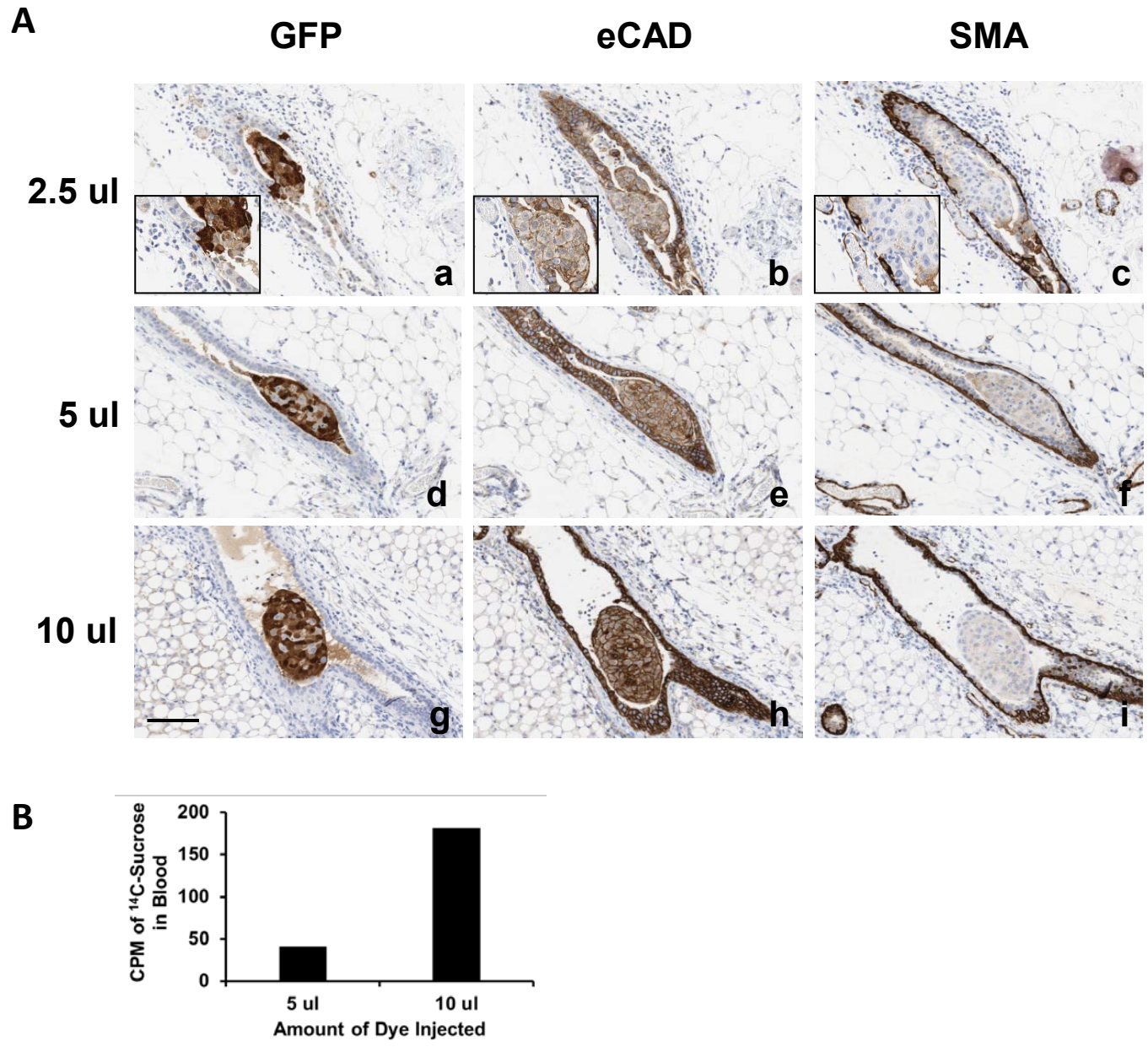
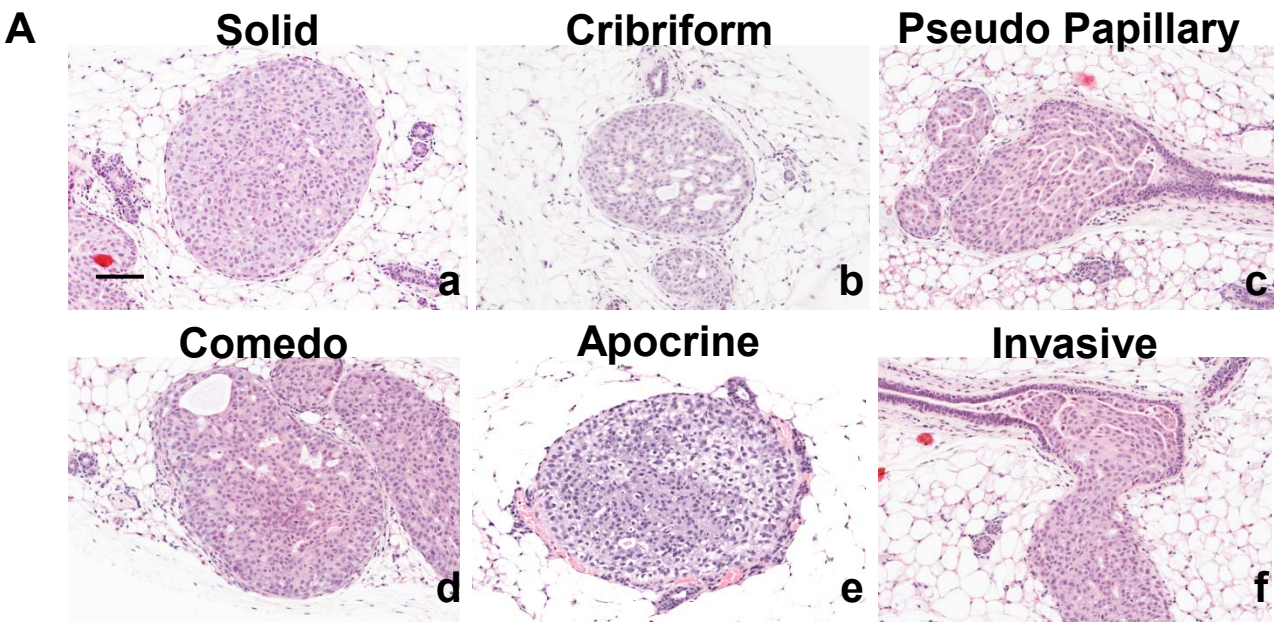
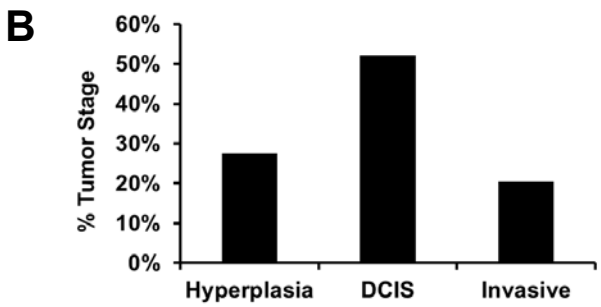
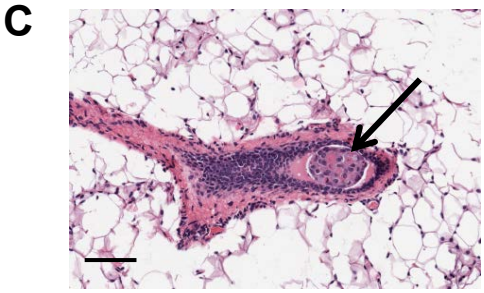
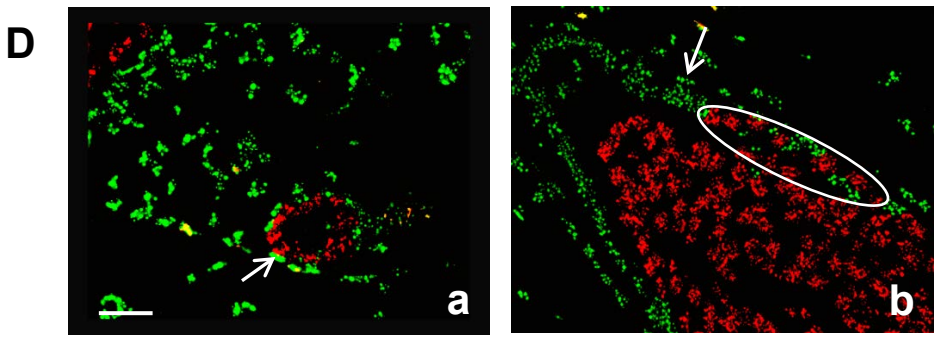

A
GFP
eCAD
SMA
2.5 ul
5 ul
10 ul
a
b
c
d
e
f
g
h
i
B
CPM of 14C-Sucrose in Blood
200
150
100
50
0
5 ul
10 ul
Amount of Dye Injected

Figure 3


A
Solid
Cribriform
Pseudo Papillary
a
b
c
Comedo
Apocrine
Invasive
d
e
f


B
% Tumor Stage
60%
50%
40%
30%
20%
10%
0%
Hyperplasia
DCIS
Invasive


C


D
a
b

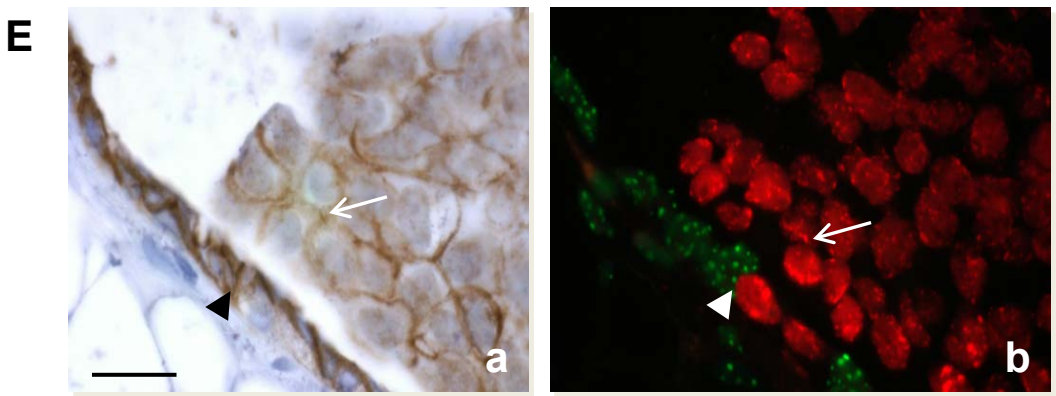

E
a
b

Figure 4

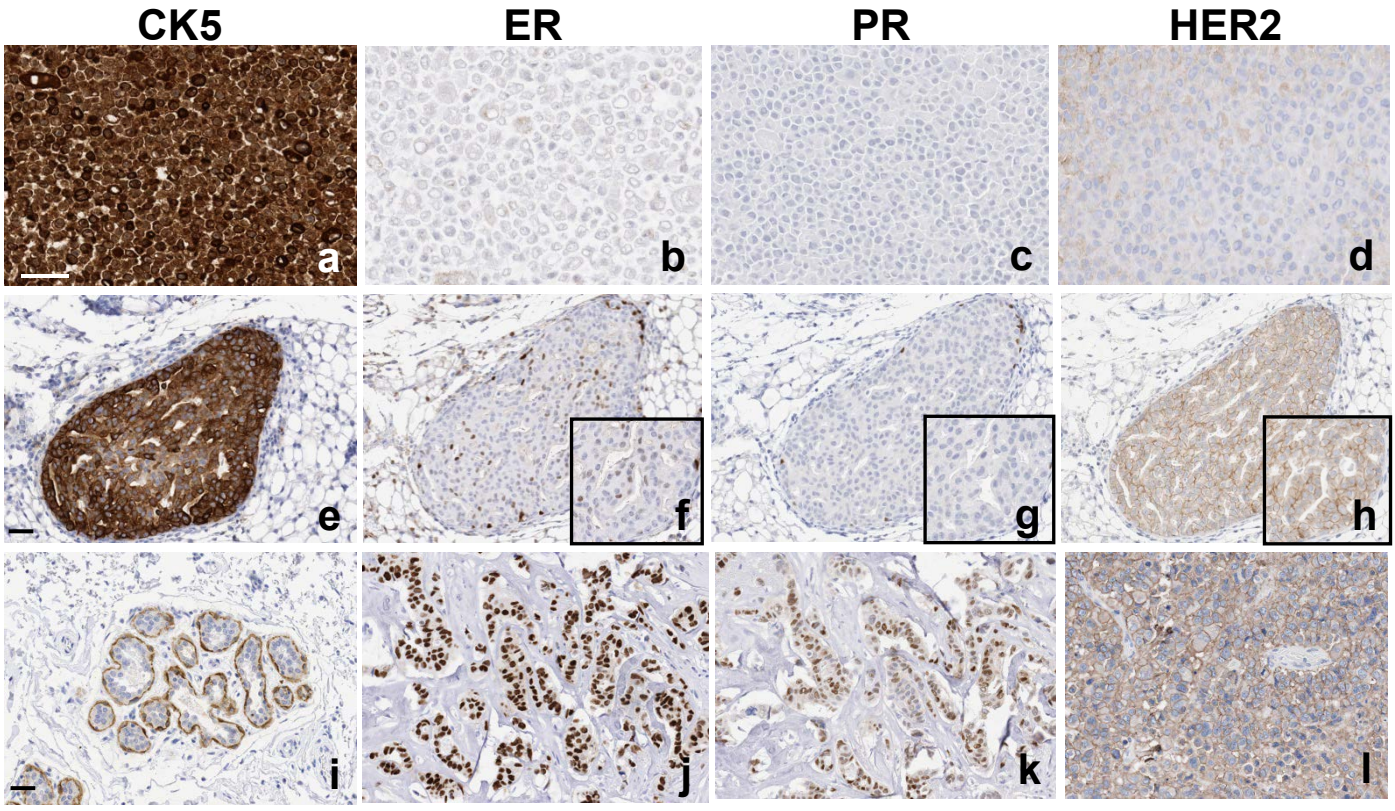
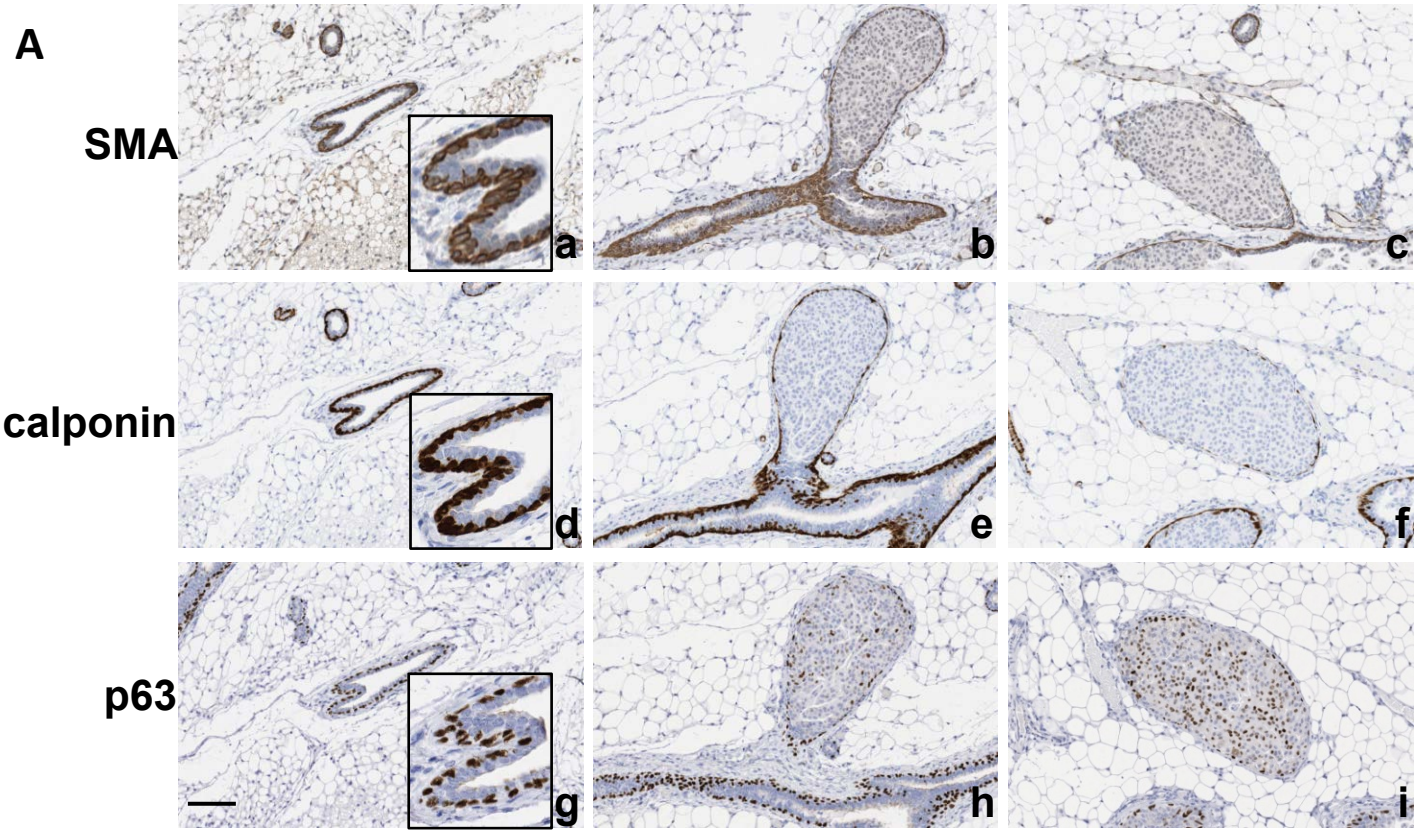


Figure 5


A
SMA
calponin
p63
a
b
c
d
e
f
g
h
i

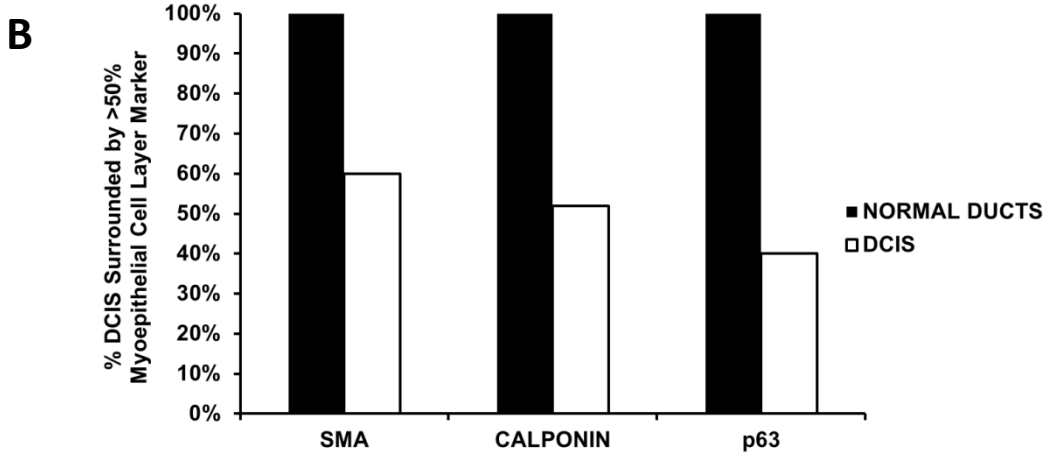
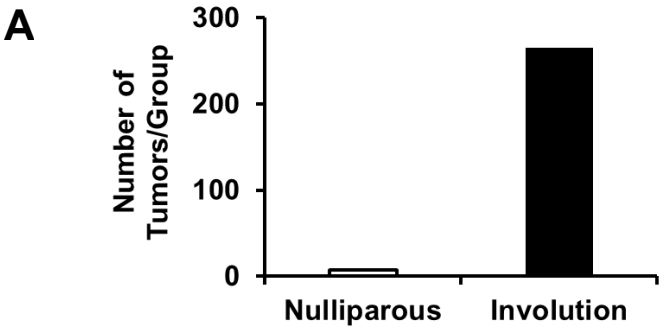
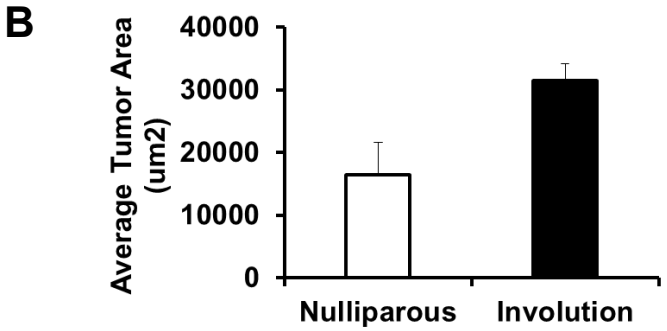
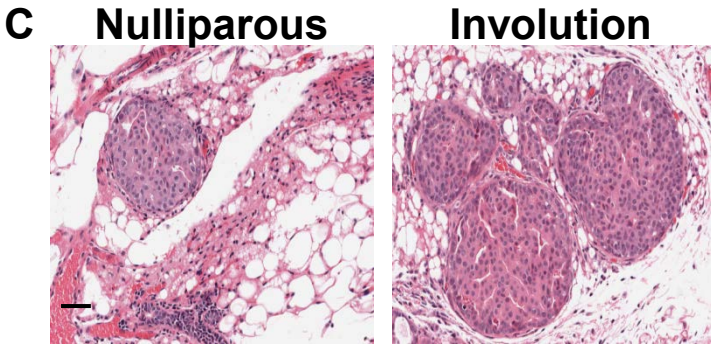

B
% DCIS Surrounded by >50% Myoepithelial Cell Layer Marker
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
SMA
CALPONIN
p63
NORMAL DUCTS
DCIS

Figure 6


A
Number of Tumors/Group
300
200
100
0
Nulliparous
Involution


B
Average Tumor Area (um2)
40000
30000
20000
10000
0
Nulliparous
Involution


C
Nulliparous
Involution

UNIVERSITY OF COLORADO DENVER
ANIMAL CARE AND USE COMMITTEE

Disposition Form
Amendment to Protocol

Protocol #	**#72110(07)1E**
Protocol Title	"Development of Preclinical Models for the Study of Pregnancy-Associated Breast Cancer and Lactation"
Investigator	Dr. Pepper Schedin
Box #	8117
Category(s)	"E"
Species	Mouse
Amendment Approval Date	05/22/2012
Protocol Expiry Date	07/21/2013

Dear Dr. Schedin,

The requested amendment to the above protocol [to add the implantation of estrogen and placebo pellets and a luminal A cell line, T47D. 528 additional animals are requested.] was considered by the Animal Care and Use Committee and has been approved.

You have been approved to work with a hazardous agent(s). Prior to initiating work with the agent(s), you **MUST** inform the Facility Manager, have an approved SOP in place, cages must be flagged, and the appropriate waste stream containers must be available. The SOP template is available at from the facility managers.

_____

Peter J. Koch, Ph.D.
Chair, Animal Care and Use Committee

Pepper Schedin[1], Traci R. Lyons[1], Jenean O'Brien[1], Eryn Callihan[1], Tanya Russell[1], Holly Martinson[1], Aik-Choon Tan[1], Kirk Hansen[1], Patricia J. Keely[2], and, Virginia Borges[1]. [1]Univ. of Colorado Anschutz Medical Center, Aurora, CO; [2]Univ. of Wisconsin, Madison, WI

Mammary gland involution drives breast cancer progression though collagen and COX-2

Utilizing a young women's breast cancer cohort from the University of Colorado Cancer Center, we show that women diagnosed as late as five-ten years postpartum have worse prognosis than nulliparous women or women diagnosed during pregnancy, and represent ~40% of all young women's breast cancer patients. We propose that the transient event of breast involution following pregnancy durably promotes early stage lesions and accounts for the poor prognosis of breast cancers diagnosed postpartum. Characterization of postpartum involution identifies tissue remodeling programs that share similarities with microenvironments known to promote metastasis. Using SHG imaging, in the involuting gland we find fibrillar collagen bundles with radially-aligned fibers similar to those observed in invasive tumors. By immunohistochemistry and FACS, we find high levels of macrophages with an M2-like profile similar to tumor-associated macrophages. In three independent mouse models for post-partum breast cancer, postpartum mammary gland involution is a driving force for cancer progression. Mammary tumors arising in the mouse involuting microenvironment express COX-2 and isolated tumor cells are motile and invasive in a collagen-1/COX-2 dependent manner. To determine whether involution-macrophages could be targeted for prevention or treatment of postpartum breast cancer, macrophages were depleted from actively involuting glands using a previously described mouse transgenic model. Surprisingly, macrophage depletion during involution blocked programmed cell death of the mammary epithelium, demonstrating that interventions targeting macrophages need to proceed with caution. Conversely, inhibition of COX-2 with celecoxib, aspirin or ibuprofen did not interfere with postpartum lobular regression. In a xenograft breast cancer model using MCF10A.DCIS cells, COX-2 inhibition did decrease tumor growth, local tumor cell dispersion and lung metastasis. NSAID treatment also suppressed collagen and tenascin-C deposition in the involuting microenvironment, suggesting that modulation of extracellular matrix proteins may be a novel mechanism by which NSAIDs exhibit chemopreventive activity. Our studies indicate two distinct but inter-related roles for COX-2 in the postpartum setting. COX-2 activity within the tumor cell is required for cellular invasiveness and COX-2 activity in the host promotes collagen fibrillogenesis and ECM remodeling, which provide a permissive environment for tumor cell invasion.

Several correlative observations implicate the collagen/COX-2 pathway in postpartum breast cancer in women: involuting breast tissue has increased collagen with radially aligned fibers, analysis of 11 publically available microarray data sets shows high COL1A and COX-2 independently correlate with decreased relapse free-survival in young breast cancer patients, and COX-2 protein is observed in DCIS lesions in postpartum cases at higher levels than nulliparous cases.

In summary, our studies suggest further research into COX-2 inhibitor use might provide a novel strategy to improve the prognosis of young women should they be diagnosed with postpartum breast cancer. The question of whether an NSAID based intervention study could be aimed at recently pregnant women at high risk for breast cancer also remains to be determined, but is an extremely desirable objective given that the ~ 6 million pregnancies in the US per year.

Supported by grants from DoD Synergistic Idea Awards BC060531 & BC10400/001, Komen Foundation KG090629, DoD Idea Award BC074970 to PJK, ACS New England Division Postdoctoral Fellowship Spin Odyssey PF-08-257-01-CSM to TRL and DoD Predoctoral Grants BC073482 to JO and BC100910 to HM.